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BIOLOGICAL BULLETIN

THE FERTILIZATION–REACTION IN *ECHINA-RACHNIUS PARMA*. VI.

THE NECESSITY OF THE EGG CORTEX FOR FERTILIZATION.

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If we define fertilization as an instantaneous irreversible reaction at or in the cortex of the egg between an ovogenous substance, fertilizin, and the spermatozoön, it must follow (1) that an egg, once fertilized, is incapable of response to additional insemination, and (2) that fragments of fertilized eggs are likewise incapable of fertilization. If, moreover, the fertilization-reaction be limited to the cortex, then it must likewise be shown (3) that uninseminated eggs, or fragments thereof, devoid of cortex are not fertilizable. The present paper aims to set forth certain observations made at the Marine Biological Laboratory, Woods Hole, Mass., on the egg of *Echinarachnius parma* which indicate that fertilized eggs, or fragments thereof, are unfertilizable; and that uninseminated eggs, or fragments thereof, devoid of cortex are likewise unfertilizable. It is therefore concluded that the cortex of the uninseminated egg is necessary for fertilization.

II.

The fertilized egg does not react to additional insemination; sperm do not enter fertilized eggs. In order to test the validity of this generally accepted statement, fertilized eggs after removal of their membranes have been inseminated two, three, and four minutes after insemination and at later stages during development to gastrulation. Such eggs have been sectioned.

In no case have sperm been found in the egg or blastomeres after even the heaviest insemination. Rupture of the blastomeres

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with outflow of cytoplasm does not facilitate sperm entry. To off-
set the possibility that fixation might be a source of error, most
diverse fluids were used. In the living egg, in addition, it was
easy to see that spermatozoa do not react with fertilized eggs.
There is here, certainly, no evidence in support of Kohlbrugge's
results.

Many experiments have likewise been made thus: Eggs are
lightly inseminated and at five-second intervals up to the time of
membrane separation are given an additional heavy insemination.
Such eggs fail to reveal polyspermy in higher per cent. than eggs
that have but one insemination.

Thus, June 28, 1918, eggs of *Echinarachnius* were inseminated
and at five-second intervals up to time of membrane separation
were reinseminated. After membrane separation, the eggs were
gently shaken to remove the membranes; samples of these were
reinseminated at six, thirteen, sixteen, and twenty minutes after
the original insemination. Samples of these eggs were fixed in
corrosive sublimate-acetic two minutes after each insemination.
No evidence of a reaction was found in any of the sectioned
material.

In addition, the bulk of the evidence (Lillie, '19) shows that
artificially activated eggs fail to react with sperm.

Wilson found that fragments of fertilized eggs of *Cerebratulus*
are incapable of fertilization. Conklin has shown that the un-
usually large polar bodies produced by the egg of *Crepidula*
through centrifugal force do not fertilize. Since in this egg polar-
body formation follows insemination, Conklin's results are capable
of the interpretation that here, too, parts of fertilized eggs do not
refertilize.

In *Echinarachnius* the situation is the same. If inseminated
eggs of *Echinarachnius* be gently shaken in a vial with bits of
broken coverslips and the fragments thus obtained be divided into
two lots, one of which is inseminated, the per cent. of development
in the two lots is the same. The insemination of these fragments,
even if made *twenty seconds* after the insemination of the intact
eggs, does not increase the per cent. of development.

These observations indicate that fertilization is irreversible, eggs

completely activated can not respond to additional insemination; and fragments of inseminated eggs behave similarly.

Through the well-known experiments of the Hertwigs, Boveri, Morgan, and others it has been shown that enucleated fragments from uninseminated eggs of sea-urchins are fertilizable as are nucleated fragments. Wilson has shown that the enucleated fragments taken from the egg of *Cerebratulus* after dissolution of the germinal vesicle are fertilizable. In a very cautiously worded paper appearing posthumously Boveri maintained his original position as to the fertilizability of enucleated fragments of uninseminated eggs.

I find that fragments from uninseminated eggs of *Echinarachnius* obtained by gently shaking the eggs in a vial with bits of a broken coverslip are capable of fertilization and development. The development of these fragments does not depend upon the presence of the egg nucleus. Some fragments without egg nuclei fail to respond to insemination. A fragment of large size and with a nucleus may not fertilize. Very small fragments with no egg nucleus develop. *I believe that the failure of fragments to fertilize is due to the absence of cortical material.* This belief is based on results which may now be considered.

III.

Toward the end of the season of 1917 I frequently found that fertilized eggs of the *Echinarachnius* gave rise to abnormal gastrulæ which I took to be ordinary exogastrulæ. They prove to be gastrulæ with masses of undifferentiated protoplasm attached.[1] The breaking up of these masses simulates cleavage. A careful study of these eggs was made and the history of this condition revealed.

I found that among various lots of eggs kept for some time in shallow dishes with little sea-water were some eggs which on return to a larger quantity of normal sea-water underwent a fragmentation. Under the microscope this process is easily followed. The eggs give off a bud or form an exovate that slowly increases in size and drops off. Thus in a given lot of eggs there are in-

[1] These masses are always located at the vegetative pole. This may be significant for the problem of polarity.

numerable cases each with a bud of varying size still attached to or just detached from the egg. If insemination takes place before the bud drops off, a membrane separates from the "egg" and never from the bud. Repeated observation puts this statement beyond doubt. I have never seen two membranes on such eggs, nor a single membrane enclosing both egg and bud. The portion within the membrane alone cleaves and develops. The portion outside the membrane never develops; it remains attached to the gastrula until completely disintegrated. In some cases the bud is so much larger than the "egg" that membrane separation takes place from a relatively small disk; the cleavage of such eggs is discoidal; such eggs never give rise to swimmers.

If the observer inseminate eggs after the buds drop off, only one member of a pair separates a membrane, cleaves, and gastrulates, though it may be the smaller. The presence or absence of the egg nucleus is of no consequence for the development of these fragments.

Any one of three possibilities was thought of as responsible for this phenomenon of bud formation in the egg of *Echinarachnius:* (1) staleness of the eggs, (2) the presence of blood, (3) the general deterioration of the sexual products toward the end of the season. Accordingly, in 1918 attempts were made to ascertain which of these factors is responsible for this bud formation. And we may state at the outset that though each factor may contribute to the production of buds, *the essential factor is the hypertonicity of the medium.*

If eggs of *Echinarachnius* are allowed to stand in sea-water for several hours, they slowly undergo changes that eventually lead to their complete disintegration. A portion of these eggs upon insemination separate membranes many of which are stuck to the swollen cortex. If previous to insemination these eggs be gently shaken, buds are formed from those with swollen cortex. Such inseminated eggs with buds separate membranes only from the "eggs" and never from the buds. These eggs cleave and gastrulate, but the per cent. is always low. Late in the season buds are more easily produced. And throughout the season the presence of blood increases the number of buds formed.

By far the easiest method for the production of a high per cent.

of buds at any time during the season is to allow the uninseminated eggs to stand in a small quantity of sea-water in a shallow dish, thus permitting evaporation; or, better, to place uninseminated eggs in hypertonic sea-water (6 parts of $2\frac{1}{2}$ M NaCl plus 50 parts sea-water). On transfer of the eggs to normal sea-water they are gently shaken or squirted through a pipette. Large numbers of such eggs produce buds.

On insemination membranes separate from but one component of these budded eggs. Only that portion of the egg within the membrane divides and gastrulates. The gastrulæ swim attached to the undifferentiated mass of budded cytoplasm which eventually disintegrates. The process of bud formation is easily followed under the microscope and insemination made at any stage. Insemination made after complete separation of the bud gives the same result: in any two given masses of egg material separated by constriction of a bud one only develops, regardless of size or the presence of the egg nucleus.

The explanation of these results on budded eggs of *Echinarachnius* is as follows: The cortex of the eggs changes under various forms of treatment. As the uninseminated egg of *Echinarachnius* lies in sea-water it slowly deteriorates. A distinguishing mark of this deterioration is the physical change in the cortex: the cortex is thick and practically transparent. Late in the season also many eggs are found with thick cortices. Blood, too, will frequently hasten this change in the cortex. Now, hypertonic sea-water very clearly brings about a physical change in the cortex. After exposure to hypertonic sea-water the cortex may be readily seen as a thick jelly-like hull enclosing the egg. It is from this jellied cortex that the membrane separates on insemination.

If an egg with a thick cortex be gently shaken on transferal to normal sea-water, the cortex breaks and the contents of the egg flows out. Indeed, merely the transfer from hypertonic sea-water to normal sea-water will tend to produce this outflow in some eggs, as they rapidly take up water. The bud is thus made up of endoplasm and is without cortical material. In favorable cases this is readily determined.

And only that component of the budded egg which has the clear rim of cortex is fertilized on insemination as revealed by the pres-

ence of the membrane, cleavage, and gastrulation. The naked mass of endoplasm rounds up still attached to the developing egg. It never reacts with sperm whether inseminated while attached to the egg or after separated from the egg. Thus the presence of the egg cortex is necessary for fertilization. Many observations make this interpretation certain.

In the egg of *Arbacia* the results are the same; indeed, if anything, they are more clear-cut.

Hypertonic sea-water is not the only agent that will bring about this outflow of endoplasm. Frequently shaking will bring it about in a few eggs of a given lot. Hypertonic sea-water is best, however. first because it produces a high per cent. of budded eggs, and second because it makes very clear that the cortex is on the egg and not on the endoplasmic mass.

One additional method may be mentioned now because its use has in turn led to some interesting experiments along another line. This method involves the use of bolting silk, soft filter paper, and lens paper. We may briefly consider this method.

Uninseminated eggs of *Echinarachnius* are dropped on bolting silk (in focus under low power of the microscope), the mesh of which has a diameter less than that of the egg, stretched above the surface of sea-water in a stender dish. If the concentration of eggs in the drop of sea-water is just right, some eggs rupture as they flow through the meshes of the silk. If the observer work rapidly, he can after trial inseminate these eggs just as they burst. The silk is then quickly thrust into the dish of sea-water. Some of the eggs form membranes with naked buds attached.

With filter paper the method is much the same. Soft moist filter paper on which is placed a drop of eggs is mounted under the microscope above sea-water in a low stender dish. The eggs flow beneath the fibers of the filter paper and thus burst because of pressure and slight drying. As they burst they are inseminated and the paper plunged into sea-water. Some of these eggs later show buds without membranes attached to cleaving eggs within membranes. Intact eggs inseminated among the fibers of filter paper in sea-water on insemination will develop normally. I have kept such eggs through to the pluteus stage. With lens paper one may obtain much the same results; the lens paper, in addition, is

much easier to handle: the endoplasmic outflow is more readily followed.

With a little care one may induce flow of endoplasm through the cortex. The naked endoplasm rounds up and in appearance is like the remaining part of the egg. But the endoplasm does not fertilize; it fails to react with sperm.

Here, again, eggs of *Arbacia* give comparable results.

While my observations were under way in 1918, Dr. Robert Chambers informed me that by the method of microdissection he was able to remove the cortex from the egg of the starfish (Lillie, '19). Such eggs are incapable of fertilization. Portions of the egg with cortical material, on the other hand, readily fertilize.

We may conclude from these observations that certain forms of treatment so alter the cortex as to facilitate endoplasmic outflow. By such treatment the fertilization capacity of the egg is not lost; it is, however, localized in only that part of the egg enclosed by cortical material. It thus follows that the inner substance of the egg is non-fertilizable in fertilized eggs not because of progressive centripetal changes set up at the cortex on insemination, but because the endoplasm is inherently non-fertilizable. Again, it is not necessary to postulate that the development of fragments from uninseminated eggs following fertilization may be due to the presence of some nuclear material of the egg (cf. Boveri). If the interpretation of the observations here reported be correct, fragments of uninseminated eggs, whether nucleated or not, are fertilizable if they possess cortical material. The egg cortex is thus necessary for the fertilization-reaction.

IV.

In any attempt at defining fertilization we must consider several facts.

In the first place, animal ova vary with respect to the stage in their development in which they are fertilized. Thus some reach the fertilizable condition before the germinal vesicle breaks down, others in the mesophase of the first maturation, still others during the second maturation, and many after maturation is complete. Starfish eggs may be fertilized at any time from the dissolution of the germinal vesicle to a short time after complete maturation.

Nor, again, is mere sperm penetration fertilization, since sperm normally penetrate ova (*Dinophilus, Saccocirrus,* etc.) some time before fertilization ensues. There are thus all possible types of fertilization with respect to the maturation stage of the egg when normally inseminated. No definition of fertilization is worth while if based on one type of egg alone.

In the second place, though the end result of fertilization is cleavage, there are here, too, many differences among animal ova. Thus the zygote nucleus may at first divide without cytoplasmic division (*Renilla*); the germ nuclei may fuse or appose merely; the cleavage spindle may be homodynamic, or heterodynamic; the sperm amphiaster may be homo- or heterodynamic, its second aster arising before or after union with the egg nucleus; and the cleavage centers may arise about the sperm nucleus or the egg nucleus or in part about each. A definition of fertilization in terms of the behavior of the germ nuclei or of the origin of the cleavage centers is manifestly inadequate.

If, for example, we consider the classic theory of Boveri that fertilization is due to the introduction of centrosomes by the spermatozoön, we realize its inadequacy at once, since it demands that the middle-piece enter the egg. It is true that the whole spermatozoön enters certain eggs whose maturation spindles are without centrosomes or asters, thus apparently supplying a deficiency. But in many other cases the middle-piece does not enter the egg, and where it does as in echinid ova the identity of its so-called centrosomes is wholly mistaken. To support the Boveri hypothesis we must shift the position of the potent centrosomes to fit those cases where the middle-piece does not enter the egg, or on entering takes no part in aster formation.

Because of failure to recall these simple facts purely morphological theories of fertilization fail. Indeed, many studies on fertilization are but studies of cell division; they deal with structures and phenomena in cell division in no wise restricted to egg cells. Nor yet have many physical or chemical theories been more fortunate. These theories are based on the study of physiological changes incident to cell division. But cell division is not fertilization.

An approach to the fertilization problem can be made only

through study of fertilization in the most diverse types of ova and by rejection of the incidental phenomena for the basic and common. What is the common factor in fertilization? So far as we know, it is some type of cortical change. But by cortical change we do not mean that the sign of the thing is the thing itself. Thus membrane separation in the sea-urchin egg is an easily visible sign of cortical change. Membrane separation, however, is not fertilization. It is here that an error lies in much of the work on experimental parthenogenesis.

Though we may doubtless gain knowledge of the nature of the cortical changes following insemination through study of these changes experimentally induced, we can not rely wholly on such work to explain fertilization. A far more simple mode of attack is to study fertilization itself. And if, in addition, the theory of the action of the agent in experimental parthenogenesis is erroneous, such a theory for fertilization can but hinder the solution of our problem. If it be true that cell division is not fertilization, it is equally true that experimental parthenogenesis is not fertilization. We must, therefore, study the fertilization process itself, the common factor of which is some kind of cortical change.

The evidence at hand indicates that the cortical changes in fertilization are due to an instantaneous, irreversible reaction between an ovogenous substance, fertilizin, and the spermatozoön. Stated in these terms the theory almost demands that the cortex is necessary for fertilization. The evidence herewith submitted points to this conclusion. The primary, if not, indeed, the whole event in fertilization, is the cortical reaction. The succeeding events with concomitant physical and chemical changes leading to cell division and development are the consequence of a complete cortical reaction between fertilizin and spermatozoön.

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THE FERTILIZATION–REACTION IN *ECHINA-RACHNIUS PARMA.* VII.

THE INHIBITORY ACTION OF BLOOD.

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The present communication aims to set forth results of experiments made during two seasons (1919 and 1920) at the Marine Biological Laboratory, Woods Hole, Mass., to test that part of Lillie's fertilizin theory which postulates that blood (in *Arbacia*) inhibits fertilization through intervention of the fertilizin and the egg (Lillie, '14). The present writer was firmly of the opinion that this postulated action might be merely a surface effect: that despite the agglutination of *Arbacia* sperm, by *Arbacia* egg-water in the presence of specific blood, the main action of the blood is on the surface of the egg so that sperm can not enter. The results of the experiments here reported, however, show that, in the egg of *Echinarachnius parma* at least, this is not the case: blood blocks fertilization in this egg by interfering with the reaction of fertilizin and egg and not with the sperm and fertilizin at the surface of the egg. For repeated observations reveal that both in straight and cross fertilization, with *Arbacia* sperm, eggs of *Echinarachnius* inseminated in blood, though they fail to develop, nevertheless take in sperm. We may divide the experiments into two groups: those that deal with straight fertilization and those that deal with cross fertilization with *Arbacia* sperm.

I.

Eggs of *Echinarachnius* obtained by cutting up ovaries in sea-water invariably give low fertilization percentages. Thus the early observations—1910, 1914, 1915—made on such eggs gave the impression that this is a poor egg for the study of fertilization. An egg suspension strained from ovaries cut up in sea-water shows a slight turbidity or greater depth in color depending upon the amount of blood and detritus present. My notes indicate that fertilizing power falls off with increasing depth of color. With shed eggs, on the other hand, the case is quite different: they invariably yield 100 per cent. fertilization. If, however, shed eggs

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of maximum fertilization capacity be inseminated in cœlomic fluid, the per cent. of cleavage is decreased. Thus equal parts of cœlomic fluid and sea-water may cut down the per cent. of cleavage to zero; higher proportions of cœlomic fluid, 75 to 100 per cent., invariably permit no fertilization.

In practice it was found extremely difficult to use large quantities of blood owing to its scarcity. Since, however, eggs from one female only were used in any given experiment, this was found no great difficulty, since the number of eggs used was very small in each case.

The method used is about as follows: Equal parts of cœlomic fluid and sea-water made solution No. 1. To half of No. 1 was added a like quantity of sea-water to make No. 2. Thus a series of half dilutions was made. One half of the last member in the series was discarded in order that all numbers would contain the same quantity of solution. Uninseminated eggs were placed in each solution—one drop of an egg suspension to each. Likewise a drop of uninseminated eggs was placed in normal sea-water equal in amount to that of mixture of cœlomic fluid and sea-water. The eggs in all dishes were then inseminated with the same amount of sperm from one male. In general, inseminations were made first in 100 per cent. and in 50 per cent. blood. Unless these gave high percentages of inhibition, I made no further dilutions.

The appended summary (Table I.) gives the results of six experiments made in 1919:

TABLE I.

THE INHIBITORY EFFECT OF SPECIFIC BLOOD ON FERTILIZATION OF THE EGG OF
Echinarachnius parma AS REVEALED BY THE PER CENT. OF
CLEAVAGE IN VARIOUS CONCENTRATIONS OF BLOOD IN
SEA-WATER IN 6 EXPERIMENTS OF 1919.

No.	Per Cent. of Blood in Sea-water.	Per Cent. of Cleavage.					
		Exp. 1.	Exp. 2.	Exp. 3.	Exp. 4.	Exp. 5.	Exp. 6.
1.....	100	0	0	0	0	0	0
2.....	50	0	0	0	0	0	0
3.....	25	0	0	0	3	6	0
4.....	12.5	0	1	0	8	33	0
5.....	6.25	0	12	16	14	50	0
6.....	3.125	49	40	47	33	81	0
7.....	1.5625	89	64	78	90	90	0
8.....	0 (control)	99	100	98	98	99	36

It is thus seen that eggs of high fertilization capacity fail to fertilize if inseminated in the presence of certain concentrations of blood. Not all eggs give results comparable to those in the table. Thus during May, 1921, several samples of blood tested showed very weak inhibitory power. In essentials, however, the results are quite comparable to those obtained by Lillie in his study of fertilization in *Arbacia*. Moreover, Lillie's interpretation of the mode of action of the blood inhibitor is sustained by this work on the egg of *Echinarachnius,* as will now be shown.

Sperm of *Arbacia* readily agglutinate in mixtures of *Arbacia* egg-water and blood as though the blood were absent. Lillie thus concluded from this that the blood does not block the reaction between the sperm-agglutinating substance (fertilizin) and the sperm; the block comes between fertilizin and substances in the egg. But it is at once apparent to the reader that this is not wholly conclusive: the substance in blood that inhibits fertilization may well do so by some action on the surface of the egg rendering sperm attachment and penetration impossible. Thus it might well be that sperm in the presence of blood and egg-water rich with sperm agglutinin of high power agglutinate; but in ordinary insemination this amount of agglutinin is not present, nor is the insemination made as heavy as the sperm suspensions must be to detect the presence of agglutinin. In the inseminations usually employed for fertilizing eggs agglutination of spermatozoa does not occur; instead, the spermatozoa stick to the egg. As a matter of fact, sperm likewise stick to *Echinarachnius* eggs inseminated in blood. The failure of such eggs to fertilize can not, therefore, be attributed to the effect of blood in blocking the agglutination of sperm to the egg.

At first I considered this result as due to the poor quality of the sperm; that it was not so much an inhibition by blood as a failure of fertilization. Subsequently it was found repeatedly that on inseminating in the presence of blood spermatozoa are attached to the eggs. Thus we have evidence to support the postulate offered by Lillie as to the mode of action of blood inhibitor. This is brought out again in the next group of experiments.

II.

It has been shown (Just, '19) that the fertilization of eggs of *Echinarachnius* by *Arbacia* sperm is greatly facilitated by the use of alkali or by heavy insemination. Though giving a lower per cent. of cleavage than alkali, heavy insemination was for several reasons the method adopted in the experiments made to determine the effect of *Echinarachnius* blood on fertilization by *Arbacia* sperm. That this cross is inhibited by blood was suggested in the earlier work. The experiments now cited indicate that this is true. I cite four experiments made in June and in August, 1920.

Uninseminated eggs of *Echinarachnius* are washed in sea-water and allowed to settle. Five drops of this suspension is distributed equally among five dishes as follows: *Lot A,* uninseminated in sea-water; *Lot B,* inseminated in sea-water with *Echinarachnius* sperm; *Lot C,* inseminated in 3 per cent. *Echinarachnius* blood with *Echinarachnius* sperm; *Lot D,* heavily inseminated in 3 per cent. *Echinarachnius* blood with shed *Arbacia* sperm; *Lot E,* heavily inseminated in sea-water with shed *Arbacia* sperm. The results follow:

Lot.	Per Cent. of Cleavage.			
	June 22.	June 24.	August 4.	August 5.
A	0	0	0	0
B	99	100	78	81
C	74	69	54	49
D	0	0	0	0
E	43	34	21	17

In some cases a concentration of *Echinarachnius* blood that has *no* effect on the fertilization of *Echinarachnius* eggs by its own sperm will not give a single membrane or cleavage with heavy *Arbacia* sperm suspension. Again, shed eggs of *Echinarachnius* are superior to eggs cut out of the ovaries for cross fertilization. Also, after thorough washing, eggs cut out of the ovaries and fertilized with *Arbacia* sperm yield a higher per cent. of cleavage. I interpret these facts as indicating that it is the presence of blood that makes cross fertilization difficult. Blood thus acts in cross fertilization as it does in straight fertilization; the differences are quantitative only. For *Arbacia* sperm enter *Echinarachnius* eggs

in the presence of blood, but they set up no reaction. In my 1917 series of *Echinarachnius* eggs, heavily inseminated with *Arbacia* sperm, this is clearly shown in the sectioned material. Moreover, these eggs are viable; though literally studded with *Arbacia* sperm, they are capable up to twenty-four hours later on insemination with *Echinarachnius* sperm of giving development of a high degree of normality.

It should be noted that in these experiments clean shed sperm of *Arbacia* was used. If the sperm be obtained from the testes admixed with blood, the per cent. of cross fertilization is reduced. This has been repeatedly observed. In some cases, indeed, the shed sperm may give around 30 per cent. fertilization and the sperm from the testes no fertilization. I believe that this is due to the toxicity of *Arbacia* blood. This toxicity is well known from Lillie's observations. I have likewise mentioned elsewhere that *Arbacia* blood is markedly toxic for *Nereis* eggs.

Similarly, mixtures of shed *Arbacia* sperm and shed *Echinarachnius* sperm exhibit no antagonism; eggs of either form or of both dropped into such sperm mixtures fertilize. With mixtures of sperm cut from the testes the results are different, for such mixtures cut down the per cent. of cleavage. In one experiment made with mixtures of shed *Arbacia* sperm mixed with shed *Nereis* sperm there was no sperm antagonism, since eggs of each form developed upon inseminations from the mixture.

These, then, are the results of inseminating eggs of *Echinarachnius* in its body fluid or own blood.

We may conclude: (1) Blood blocks straight fertilization. (2) Blood blocks cross fertilization. (3) Blood blocks both straight and cross fertilization after the spermatozoa stick to the eggs or enter them and not by preventing the attachment of spermatozoa to the eggs.

These conclusions admit of certain suggestions concerning the nature of specificity in the fertilization reaction. We may discuss these briefly.

III.

The block to cross fertilization is cortical. As Lillie says: " The various methods used to induce hybrid fertilization—staling of

eggs, high concentration of sperm, use of alkalies or other chemi-
cals—have therefore this one feature in common, that they destroy
the chemical or physical integrity of the cortex of the egg " (Lillie,
'19, page 219). Specificity in fertilization thus manifests itself in
the cortex of the egg.

But specificity in fertilization is not absolute, but relative. This
fact would seem to indicate that the results of straight and of
cross fertilization are due to quantitative, not qualitative, differ-
ences in the cortical response to insemination; species sperm more
readily than foreign sperm overcome the same resistance to ferti-
lization set up by some cortical substance or condition. The ques-
tion, therefore, comes down to this: What in the cortex is re-
sponsible for the block to fertilization, whether by species or
foreign sperm?

In the first place, most methods used to induce cross fertilization
in echinids hasten the loss of fertilizin. Thus staling is an easy
method for the removal of fertilizin. Eggs allowed to stand or
repeatedly washed lose their fertilizin content. Washing the eggs
rapidly with dilute sea-water brings about a loss of fertilizin.
Dense sperm suspensions rapidly bind available fertilizin. I ven-
ture the opinion that heat hastens the loss of fertilizin also.

If, now, we postulate that specificity in fertilization is wholly
due to the presence of fertilizin, then must we also take the next
step, namely, that cross fertilization is most successful when the
fertilizin is reduced? That is, fertilizin is necessary for straight
fertilization, but a block to cross fertilization; certain kinds of
artificial parthenogenesis (heat, for example, on *Nereis* egg) de-
pend upon the presence of the fertilizin in maximum concentra-
tion; certain eggs lose their capacity for fertilization by species
sperm very rapidly (*Platynereis*); but with foreign sperm the case
is otherwise—it can fertilize after an egg is no longer capable of
response to artificial stimulus or that of species germ. But might
not specificity in fertilization be accounted for in part on the basis
of the data presented in this paper? This would mean at least
with the knowledge at hand that specificity in fertilization is due
in part to the blood, since the presence of blood blocks fertilization
by species or foreign sperm.

When species sperm comes in contact with an egg, it gains entrance and fertilizes against the blood present. The greater the amount of blood, the more difficult the fertilization. Indeed, the blood may actually inhibit fertilization in every egg. Therefore, dense sperm suspensions must be employed for fertilization in the presence of blood rich in inhibitor. The blood inhibitor acts by binding the fertilizin so that the fertilizin can not react with the egg receptors. Heavy insemination insures fertilization perhaps by increasing the chances of some spermatozoa locating fertilizin free of blood inhibitor. Or in heavy insemination the onslaught of numerous sperm brings it about that the fertilizin shakes free the inhibitor.

The blood slowly leaves the egg as it lies in sea-water. But the fertilizin also goes. Hence while the egg is losing inhibitor it is also losing fertilizing power. The blood is perhaps never an irremovable block to species sperm; however, though present in but a trace, it serves to block foreign sperm. In staling, therefore, what results is not only loss of fertilizin, but also loss of blood. The loss of blood makes possible cross fertilization.

What is true of staling is doubtless true of other methods for obtaining cross fertilization—heat, use of alkali, and of dilute sea-water; they serve to remove the blood block. The fertilizin remains albeit in reduced quantity. Whenever an egg is capable of fertilization it possesses the fertilizable substance. And it is safe to assume that an egg that will not respond to its own sperm will not cross fertilize.

From this point of view, then, fertilizin is not the only factor in specificity. It is specific since it engages species sperm against the inhibition of blood. But the blood is an aid to specificity, since it blocks all sperm, species sperm least of all.

LITERATURE.

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THE FERTILIZATION–REACTION IN *ECHINA-RACHNIUS PARMA.* VIII.

FERTILIZATION IN DILUTE SEA-WATER.

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The writer has shown,[2] by means of experiments made in 1920 at the Marine Biological Laboratory, Woods Hole, Mass., that a given dilution of sea-water with tap water which leaves the unin-seminated egg of *Echinarachnius parma* intact is rapidly injurious to the inseminated egg during its process of membrane separation. After membrane separation the egg is likewise resistant to the action of the dilute sea-water. Certain experiments were also made during the same season to learn if it is possible to procure fertilization and development in diluted sea-water to which the inseminated egg during membrane separation is susceptible. These experiments were repeated during May and June of the 1921 season at the laboratory with essentially the same results.

In experiments made to discover if in a given dilution of sea-water, which is injurious to the inseminated egg of *Echinarachnius* during the period of membrane separation, it is possible to fertilize the egg, it was soon apparent that cleavage and normal gas-trulation are not possible in a dilution which permits membrane separation. Thus during the period June 21 to June 28, 1921, inclusive, eggs from twenty-four females were inseminated in normal sea-water and in sea-water of varying dilutions. In all cases due precautions were taken as to the bulk of eggs, quantity of solution, and density of the sperm suspensions used in order that as far as possible conditions be made uniform. The results obtained with 95 per cent. sea-water (95 parts sea-water plus 5 parts tap water) and the dilutions ranging from this to 80 per cent. sea-water (80 parts sea-water plus 20 parts tap water) may be

[1] Zoölogical Laboratory, Howard University.
[2] Am. Jour. Phys., 1922, 61, 516.

summarized briefly as follows: In 95 per cent. sea-water eggs fer-
tilize normally as revealed by the per cent. of membranes formed.
They cleave and gastrulate in this dilution. In most cases the eggs
are scarcely to be distinguished from eggs inseminated in normal
sea-water. In 90 per cent. sea-water membranes are normal and
cleavage fairly normally; but these eggs form about 10 per cent.
exogastrulæ. In 85 per cent. sea-water the per cent. of mem-
branes is close to normal—95 per cent. or slightly more. The
cleavages show some abnormalities and the per cent. of exogas-
trulæ is greater than in 90 per cent. sea-water. In 80 per cent.
sea-water the per cent. of cleavage falls and the per cent. of
exogastrulæ increases.

The experiments indicated that the dilution made up with 75
parts sea-water plus 25 parts tap water is the lowest which permits
cleavage. In this the gastrulæ are very abnormal. We may,
therefore, turn our attention to the experiments made with this
and greater dilutions of sea-water. Eggs inseminated in dilutions
greater than those that permit cleavage separate membranes.
Even in the dilution made up of equal parts tap water and sea-
water some membranes separate, though these are often hard to
see. Eggs, therefore, will respond to insemination with at least
abortive cortical changes, though they can not cleave. I cite now
one experiment which was made—as the majority of these experi-
ments—on the eggs of three females.

June 21, 3:30 P.M. Six series of dilute sea-water, each con-
sisting of 6 members, as follows:

No.	1.	2.	3.	4.	5.	6.
Parts sea water...................	50	55	60	65	70	75
Parts tap-water...................	50	45	40	35	30	25

Equal portions of uninseminated eggs from each of 3 females
(A, B, and C) put in each dilution of sea-water to make Series IA,
IB, and IC, IIA, IIB, and IIC.

Series IA, IB, and IC uninseminated.

Series IIA, IIB, and IIC inseminated with same quantity of
sperm from one male. Inseminations in each series 5–25 seconds
after eggs in dilute sea-water.

Controls: uninseminated eggs in normal sea-water; inseminated eggs in normal sea-water.

The eggs were examined at intervals. The next day, 7:50 A.M., the uninseminated eggs (Series I*A*, I*B*, and I*C*) showed an average of less than 10 per cent. cytolysis. The history of Series II. is given in the table below (Table I.):

TABLE I.

PER CENT. OF MEMBRANES AND OF CLEAVAGE IN EGGS OF *Echinarachnius* IN-
IN SEA-WATER OF VARYING DILUTION.

No.	Amount of Sea-water in 100 Parts of Sea-water plus Tap Water.	Per Cent. of Membranes in Eggs from the 3 Females.			Per Cent. of Cytolysis Two Hours after Insemination.			Per Cent. of Cleavage 5 Hours after Insemination.		
		A.	*B.*	*C.*	*A.*	*B.*	*C.*	*A.*	*B.*	*C.*
1.................	50	0	2	1	18	4	9	0	0	0
2.................	55	0	2	0	4	2	1	0	0	0
3.................	60	7	7	7	0	3	0	0	0	0
4.................	65	5	22	20	0	1	0	0	0	0
5.................	70	14	63	47	0	3	0	0	0	0
6.................	75	60	95	87	0	2	0	5	16	4
Uninseminated control.........	100	0	0	0	0	0.1	0	0	0	0
Inseminated control	100	100	98	100	0	0.5	0	100	97	100

This experiment indicates that eggs inseminated in dilutions of sea-water may separate membranes, though they do not cleave. It would, therefore, be erroneous to assert—if membrane separation be the criterion for fertilization—that eggs that do not cleave have not been fertilized. Rather the failure to cleave is due to the action of the dilute sea-water in interfering with the cleavage mechanism—particularly with the activity of the hyaline plasma layer. There is no question here of "partial fertilization"; it is wholly a question of incomplete cleavage. This is important for the experiments that we may now consider.

In the experiments now to be considered it was repeatedly found that eggs may be inseminated in a dilution that is destructive to the egg which is inseminated in sea-water and exposed to this dilution during membrane separation. The criterion of this destructive action is the differential cytolysis of uninseminated and inseminated eggs before, during, and after membrane separation.

With cleavage, however, the case is quite different. That is, the per cent. of cleavage of eggs inseminated in a given dilution is no higher than that of eggs inseminated in sea-water and transferred to the dilution before, during, or after membrane separation. There might be a slight indication that eggs exposed during membrane separation show a lower per cent. of cleavage. I believe, however, that my figures on this point are not decisive.

I give now one experiment to show the per cent. of cytolysis in eggs inseminated in dilutions of sea-water as compared with that of eggs inseminated in sea-water and placed in the dilutions before, during, and after membrane separation.

June 27, 11:25 A.M. Following dilutions prepared:

No.	1.	2.	3.	4.	5.	6.
Parts tap-water...................	50	45	40	35	30	25
Parts sea-water........	50	55	60	65	70	75

Five dishes for each of the six dilutions containing 10 c.c. each. Thus 5 series of 6 numbers each.

Drops of uninseminated eggs from one female added to dilutions as follows:

Series 1: 1 drop of eggs in each dilution.

Series 2: 1 drop of eggs in each dilution; inseminated immediately.

Series 3: Drop of eggs to each dilution 15 seconds after insemination in sea-water.

Series 4: Drop of eggs to each dilution during the period of membrane separation 35 seconds after insemination in sea-water.

Series 5: Drop of eggs to each dilution two minutes after insemination in sea-water.

Controls: Uninseminated eggs in normal sea-water; inseminated eggs in normal sea-water.

The results of this experiment follow:

12 M.

Series 1: Less than 1 per cent. cytolysis.

Series 2: As in Series 1.

Series 3:
(12:00 M.)

No.	Dilution (Parts of Sea-water in 100 Parts of Solution.	Per Cent. of Eggs Intact.	Per Cent. of Eggs Cytolyzed.
1	50	79	21
2	55	77	23
3	60	89	11
4	65	98	2
5	70	97	3
6	75	100	0

Series 4:
(1:00 P.M.)

1	50	50	50
2	55	47	43
3	60	85	15
4	65	80	20
5	70	90	10
6	75	97	3

Series 5:
(1:30 P.M.)

1	50	69	31
2	55	83	17
3	60	86	14
4	65	97	3
5	70	99	1
6	75	98	2

Controls: Uninseminated in sea-water—1 per cent. cytolysis.
Inseminated in sea-water 99 + cleavage.

It is clear from this experiment that eggs exposed to a dilution of sea-water during membrane separation cytolyze in slightly higher per cent. than eggs inseminated in the dilution. Similarly, eggs inseminated in normal sea-water and exposed before or after membrane separation cytolyze in slightly lower per cent. than eggs exposed during membrane separation. The cleavage per cent. of *surviving* eggs is about the same.

If we hold that membrane separation is a criterion of fertilization, then eggs inseminated in a given dilution of sea-water are fertilized whether they cleave or not. This admittedly is not a strong case, but it gives some support to the position that the fertilization reaction is practically instantaneous. It is not fertilization that is checked by the action of dilute sea-water, but the reactions which set up by fertilization lead to cell division.

REACTIONS OF CELL–BODIES AND PSEUDOPODIAL FRAGMENTS OF *DIFFLUGIA*.[1]

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INTRODUCTION.

The purpose of this paper is to record a number of observations which were prompted by an incident occurring on October 7, 1919, while one of us (Kepner) was watching a *Difflugia pyriformis* Ehrenberg under the compound microscope. The animal suddenly retracted an unusually long pseudopod, and in doing so its distal end was torn off. In a short time the fragment began to show amœboid movement; meanwhile the animal sent out other pseudopods, and within a few minutes the two masses of protoplasm made contact and fusion took place. This naturally called to mind the work which had been done by others on regeneration and restitution; therefore a series of experiments were undertaken in order to find out to what extent, if any, this phenomenon was exhibited in *Difflugia*.

It is generally known that many animals possess conspicuous powers of regeneration. For example: snails are able to regenerate tentacles; earthworms, flatworms, and polyps may have their bodies severed and each fragment be able to restore the parts that it lacks to complete a body. Verworn (1892) observed that severed fragments of *Orbitolites* would occasionally adhere to the ends of parental pseudopods. Jensen (1896) pointed out that *Orbitolites* and *Amphistegina* (belonging to the order *Foraminifera*) would sometimes recover fragments belonging to themselves and make them again a part of their bodies. Wilson (1907, '11) has shown that the tissues of sponges broken up or macerated by passage through the meshes of bolting cloth, so that the component cells

[1] This is the first of a series of contributions on this subject. More extensive observations and experiments are now being conducted by the junior author with other genera of Rhizopods, as well as with the genus *Difflugia*.

are separated and distributed in water, will reassemble and form new individuals.

The conditions of life under which the above animals live impose a risk of the body parts. *Difflugia* is protected, to a certain extent, from external environments by its shell, and perhaps also by the formation of a plug of mucus about the mouth of its shell when unduly stimulated. Nevertheless, in sending out long, slender pseudopods, it, too, runs a risk of losing some of its protoplasm, for, as recorded by Verworn (1890), when the projecting pseudopods are strongly stimulated, they are frequently jerked back so suddenly as to whip off their ends.

The Organisms.

Various species of *Difflugia* were used in these experiments, all of which gave similar results. However, it was found that *D. acuminata, D. corona, D. pyriformis, D. spiralis,* and *D. vulgaris* were more favorable for study because of the nature of their pseudopodial formation, and also because they commonly drag themselves after their advancing pseudopods with the shell in a reclining position, so that the mouth of the shell and the bases of the pseudopods can be seen easily. This feature also affords a better opportunity for cutting off pseudopods without danger of striking the shell.

Methods.

(a) Collecting.

The animals were collected in small puddles in a cow pasture near the University of Virginia. These puddles were near a fresh stream and frequently obtain a supply of fresh water, due to rains, and the overflowing of the banks of the stream. In the same puddles other protozoa, fresh-water crustacea, and various algæ are also found. The brown ooze and vegetable matter on the bottom of these little pools were carried to the laboratory in fruit jars, then allowed to stand for several hours, after which the surface of the sediment, along with sufficient water to fill the vessel to a depth of approximately five millimeters, was transferred to Petri dishes. The organisms can be kept in this way for several weeks, provided fresh water is added occasionally. In these experiments no attempt was made to maintain pure line cultures.

(b) *Apparatus and Technique.*

Individuals can easily be isolated for study by placing a Petri dish, containing the organisms, under a low-powered binocular, locating the animal desired, and transferring it by means of a narrow-mouthed pipette to a plain slide containing a large drop of spring (or distilled) water. The amputations were made with fine glass needles, which can readily be drawn in a small hot flame. It is essential to use hard elastic glass for this purpose.

At first the operations were made under a binocular, and the slide containing the organism was then transferred immediately to a compound microscope for observation, but later operations were made under the compound microscope, using a 32-mm. objective and a No. 10 eyepiece (B. & L.). The latter method is altogether more satisfactory, since it enables the observer to keep sight of the performance from the very beginning, and also avoids any jarring or shifting of the protoplasms, which might take place while transferring the slide. Practically all of the observations were made under the 16-mm. objective and No. 10 eyepiece. Most of the distances given in this paper were estimated either in terms of the diameter of the field or in terms of the diameters of the shells of the individuals concerned. A later and more satis- factory method is to use a camera lucida and trace the outlines of the experimental objects at definite intervals. By this method exact record can be kept of the relative sizes and positions, as well as the paths followed by each body.

I. Is Reunion of Cell-body and Fragment the Usual Occurrence?

In order to determine this we performed approximately one hundred experiments, the majority of which gave positive results. The few negative cases observed were apparently due to one of the following causes: (a) Severe injury to cell with consequent failure on its part to extrude pseudopods, (b) separation from fragment by too great a distance, (c) evaporation of water before completion of reaction. These results have been repeatedly con- firmed by graduate students in this laboratory. Since mucus is involved in the organisms' locomotion, it occurred to us that the

separated parts might follow a mucous trail, or even invisible strands of protoplasm, and thus contact would be effected. For this reason, in most cases after a pseudopod was severed the cell-body was moved to a considerable distance, and yet the fragment was recovered. Again the cell-body has been moved in a wide detour around on another side of its fragment, after which it would approach without regard to the course along which it had been pushed. The mouth of the shell has been placed in every angle to the fragment from zero to one hundred and eighty degrees; nevertheless the fragment is usually recovered. To give the details of each experiment would require too much space and necessitate repetition of similar results; therefore, the following two observations have been selected as typical of the usual reaction: [1]

On July 24, 1920, the end of a pseudopod was cut from a *Difflugia vulgaris* by Mr. E. Paylor, of this laboratory, after which the cell-body was immediately moved 500 micra away from it, the mouth of the shell being directed away from the fragment, as shown in Fig. 1—*A*. Within a minute the animal, which had withdrawn into its shell after the operation, began to send out two pseudopods. After these pseudopods had been extended to a distance of about fifty micra, their ends adhered to the glass slide on which the animal was lying. Then by a movement somewhat similar to a hand-spring the body proper was thrown over the extended pseudopods, so that now the mouth of the shell was pointed toward the fragment (Fig. 1—*B*). The animal then began to move in the direction of the fragment, moving in a counter-clockwise curve as indicated by Fig. 1—*C, D, E,* and *F.* This movement was continuous until the fragment was encountered and reappropriated through fusion of the two masses of protoplasm (Fig. 1—*G*).

The second example given to illustrate the phenomenon of fusion is perhaps more interesting, for in this case more than one fragment was involved. In this experiment exact record of the time

[1] We realize that the conclusions drawn in this paper would be more convincing if we published our data in extenso, but since this is a preliminary report of observations on a subject which is now being actively investigated with a view to further and more extensive publications, it seems best to present our results here by means of typical experiments and general statements regarding the entire investigation.

at which each event took place was made. At 11 : 38 A.M., June 10, 1920, a small fragment, *c*, Fig. 2, was cut off from a *Difflugia spiralis*. The animal immediately withdrew ·its extended pseudopods, but not completely within its shell. It then sent out two new pseudopods, and at 11 : 39 A.M. the ends of both these were cut off, *a* and *b*. The animal was so stimulated by this second operation that it quickly retracted its pseudopods, and in doing so was turned through an angle of ninety degrees, so that now its mouth lay directed at right angles to the fragments, which were lined up in a row. In a short time the animal extended a short pseudopod which curved around alongside the neck of its shell and fused with *a* at 11 : 40½ A.M. The cell-body was then moved 600 micra away and placed so that the side of its shell was toward the remaining two fragments which had been left in their original positions. By 11 : 45 A.M. it had advanced again and fused with fragment *c*. The animal was then stimulated with a glass rod, so that it withdrew into its shell again; but by 11 : 45¾ A.M. it had sent out another pseudopod which fused with fragment *b*. Thus within seven and three quarter minutes from the time the first fragment was cut off the animal had picked up and reappropriated all three fragments, though during the interval it had been stimulated by the observer so violently as to make it contract four times, and at one time it was moved six times its shell's diameter away, through which distance it had to travel in order to get back to its missing parts.

It has not been determined exactly what the maximum range of positive response is—*i.e.*, the limit within which fusion always occurs. One positive case was observed in which the cell-body and fragment were separated by one and one half millimeters. But chance wandering might have brought the animal within the positive range. Be this as it may, chance can hardly explain the large percentage of positive observations made. When the two elements were separated by great distances, we encountered a small percentage of negative reactions. However, in every case which we observed where the cell-body and fragment were separated by a distance of 500 micra or less fusion always took place, unless the cell-body was injured. It seems, then, that reunion is

the usual thing which takes place, if the missing parts be not too far removed.

II. Is the Phenomenon a Process of Feeding?

From the experiments described in the preceding section the conclusion was drawn that *if a fragment of protoplasm be severed from a Difflugia, it is usually reappropriated.* The question naturally arises: Does the animal react to its fragment as a piece of food, or do the two coalesce? If the fragment is taken up as food, we would expect to see: (*a*) formation of a food vacuole and enlargement at site of ingestion, (*b*) a similar reaction to fragments from closely related forms, and (*c*) no sharp distinction as to whether or not the fragment showed visible signs of life, such as movement.

(*a*) In some of these observations the animals were in such a position at the time contact was made that it was impossible to see what took place on account of the shell, but in the majority of cases the view was unobstructed, so the entire process could be witnessed. The usual reaction is approximately as follows: As the advancing pseudopods of the cell-body near the fragment they usually cease to become attached to the substratum, as in the ordinary process of locomotion; thus movement on the part of the animal is slowed down. If at this time only one pseudopod is extended, smaller secondary ones may be sent out near its base or from the mouth of the shell. The primary pseudopod may make direct contact with the fragment, or it may form a small cup into which the fragment is fitted, or a niche may be formed by the extrusion of one or more secondary pseudopods, and contact is made in the niche thus formed. If there are two or more primary pseudopods, they may encircle the fragment collectively, or only one of them may take part in the reaction. When contact is made there is ordinarily a disturbance of the protoplasmic surfaces, which varies from a mild local shock to a violent contortion involving the entire (visible) protoplasm. When the "contact shock" is very great it is impossible to observe what becomes of the fragment, but when it is slight the granular ectoplasm constituting the fragment can be seen streaming into the pseudopod and

becoming disseminated, as though the fragment were a lateral branch which the pseudopod was withdrawing. There is no sign of incoherency or enlargement at this place (see Fig. 5—*A, d*).

(*b*) The reaction toward foreign fragments is different, as will be brought out later.

(*c*) On many occasions it was observed that after fragments had been separated from their cell-bodies for a long time they would cease to move, become rounded off, and take on an opaque granular appearance. The time required to bring about this change depends on several conditions, particularly on the size of the fragment; a large fragment retaining its power of movement longer, other things being equal. Though it has not been determined experimentally, it is reasonable to presume that the physiological condition of such a fragment is changed as well as its appearance; yet its molecular composition can hardly be so altered as to change its food value. Though over twenty-five observations have been made on the reaction between *Difflugia* and its fragment, which had assumed a granular, opaque, spherical condition, not a single instance was found in which the animal picked up such a fragment. The following example is illustrative of the reactions obtained:

On June 10, 1920, at 11 : 47 A.M., a large pseudopod was cut from a *Difflugia spiralis* and the cell-body was then moved out of the field. The fragment moved about for fifteen minutes, then gradually ceased and assumed a spherical shape. By 12 : 15 half of the bulk constituting this mass of protoplasm showed disintegration to such an extent that it had changed from the spherical condition and was scattered, in an uneven manner, around the portion which still retained its rotundity. At this time the cell-body was brought up and the mouth of its shell placed in contact with the fragment. The animal soon extended two pseudopods which came in contact with the fragment in the manner indicated in Fig. 3—*A*. For two minutes the animal remained practically passive, then it began to move forward, taking with it the portion of the fragment which was still in a spherical condition. This was held by a secondary pseudopod which had formed an imperfect cup around it (Fig. 3—*B*), the disintegrated portion being left behind. Movement was continued in the same direction until

the animal had traveled twice the distance of the diameter of its shell. But at 12:22 P.M. it rejected the portion of the fragment which it held and reversed its course, leaving the fragment behind.

From our observations, then, it seems evident that severed fragments are not taken up by the cell-body as food, but that the separated bits of protoplasm recombine with the original mass and take up their function as though nothing had happened, provided disintegration processes have not set in before contact is made.

III. Does Fusion Take Place at Any Point Along the Pseudopod?

A *Difflugia* progresses by extending pseudopods (sometimes they are very long and slender), the ends of which become attached to the substratum. With this in mind, one would naturally expect that the end of an advancing pseudopod would first make contact with a fragment which the animal was approaching. On the contrary, this has never been observed. In the last section attention was called to the fact that when nearing a fragment the ends cease to become attached. At this stage they usually wave around in the medium, and further progression is made either by this waving motion, or else by smaller pseudopods near the mouth of the shell. Though not all cases were quite so clear cut, the following example illustrates the principal region of a pseudopod involved:

On October 7, 1919, a *Difflugia pyriformis* was observed extending a large pseudopod toward its fragment *a* (Fig. 4—*A*). In the meantime the fragment was moving in the direction shown by *e, f,* and *g* (Fig. 4). After its end had passed well beyond the fragment, the pseudopod was bent toward the fragment until its side had come in contact with the rear end of the moving ectoplasmic body. When this contact was made, the animal threw out an intimately fitting cup around the posterior lobe of the fragment (Fig. 4—*B, g,* and *g'*). Immediately the protoplasm, which had been detached, flowed back into the pseudopod, without causing any perceptible increase in its diameter.

In all cases which we observed fusion took place along an extended mid-region, neither at the tips nor at the bases of pseudopods.

IV. Does the Fragment Take an Active Part in Restitution?

If fusion between a cell-body and its fragment is true restitution as has been claimed, one should not be surprised to see the fragment taking an active part in the process. With this in view, especial attention was given to the movements of fragments. Without some object to mark the original positions, it is very easy to confuse distances traveled by each body when two are approaching each other. Therefore, when observations were made without the use of a camera lucida to plot the positions, care was taken to avoid falling into this error. Whenever fusion took place, the protoplasm constituting the fragment seemed to flow into, and commingle with, the protoplasm of the cell-body. But it was not always possible to observe an appreciable movement on the part of a fragment toward its cell-body before contact was made. However, on many occasions this could be seen. The two following accounts are typical of the more striking reactions of this sort:

On November 28, 1919, a fragment was cut from a *Difflugia spiralis,* after which the cell-body was moved 1,500 micra distant. Immediately after separation the fragment had the shape of an elongated ovoid, then its contour changed continually for the next thirteen minutes (Fig. 5—3, 4, 5, 6, 7, 8, 9, etc.). Each figure represents the number of minutes that had elapsed since the operation took place. During the first twelve minutes its movement seemed to be indefinite, as indicated in the diagrams, but by this time the cell-body had advanced until the end of its nearest pseudopod was within 150 micra of the fragment. From this time there was a decided movement on the part of the fragment in the direction of the cell-body. As soon as the fragment had come within reach of the long pseudopod, which the cell-body was extruding, movement by the latter slowed down, and the distal end of the pseudopod was lifted. Under this the fragment passed. The fragment continued the approach, and two minutes from the time it had come within the radius of the animal's pseudopod it had traveled about twenty micra and reunited with its cell-body (Fig. 5—*A, a, b, c,* and *d*).

On November 8, 1919, a large fragment was cut from a pseudo-

pod at 10:44 A.M. This soon became greatly contorted, and from its tangled mass two amœboid fragments emerged. The water became shallow soon after the operation and it was necessary to add more, which separated the fragments a considerable distance from the cell-body. At 11:16 A.M. the fragments were placed in the vicinity of the cell-body again (Fig. 6—*A, a, b*). At 11:23 A.M. fragment *a* had shifted its axis so that it now lay directed toward the mouth of the animal's shell. Fragment *b*, during the same period, traveled to position *x*. The cell-body was now pushed up to the relative position shown at *B* without disturbing the fragment. The anterior tip of fragment *b* was immediately shifted so that it was now directed toward the new position of the shell's mouth. The entire fragment had advanced to position *y* by 11:27 A.M. At this time fresh water was added to the slide, and in doing so the fragments were lost.

That such enucleated fragments show a positive response to stimulus, whatever it be, seems unquestionable. The importance of this is, perhaps, emphasized by the fact that all fragments involved in our observations were not simply enucleated bodies of protoplasm, for endoplasm did not constitute a portion of any of them. They were, therefore, *enucleated ectoplasmic fragments*.

V. Will Two Enucleated Fragments Fuse with Each Other?

Having observed the readiness with which fusion took place between *Difflugia* and its ectoplasmic fragment, the question suggested itself—*Is there a tendency between two pieces of protoplasm to fuse when no restitution of a cell would result from such a fusion?* Not a sufficient number of experiments has been performed to warrant an emphatic statement in regard to this point. But at least twenty observations have been made in which two or more fragments from the same organism were involved. In these experiments the fragments were only ectoplasmic in composition and varied in size from five to twenty micra in diameter. The results of these experiments can be illustrated by the following example:

A large and a small fragment were cut from a *Difflugia spiralis*, after which the animal was removed from the slide. The frag-

ments were separated by the distance of about fifty micra, and for two minutes they moved around quite actively, but no progress was made toward uniting. They were then placed in contact, by using a glass needle, so that now they were in the positions shown in Fig. 7—*m*. This contact was a very close one, the adjoining surfaces being very extensive and the line of demarcation quite indistinct, yet discernible. Ten minutes later the activity of the fragments had begun to cease and they showed signs of rounding up into spherical bodies, each piece into its respective sphere (Fig. 7—*n*). After ten more minutes the spherical condition had been attained and the pieces showed only a small point of contact (Fig. 7—*o*).

Thus, after having been placed in direct contact, there was a failure on the part of these two fragments of ectoplasm to unite— though they had come from the same cell. It seems, then, from our observations, that enucleated ectoplasms do not fuse with each other.

VI. WILL *Difflugia* FUSE WITH PSEUDOPODIAL FRAGMENTS BE-
LONGING TO AN INDIVIDUAL OF A DIFFERENT SPECIES?

(a) *As Food.* (b) *By Protoplasmic Union.*

The criteria for determining whether or not the fragments serve as food have been set forth in a previous part of this paper. How-ever, since the reaction of an individual toward fragments belong-ing to closely related forms was proposed as one method for determining this, these experiments were designed to shed some light on this subject. Furthermore, it occurred to us that in such organisms an individual might fuse with foreign fragments in the same manner described for cell-bodies and their own fragments. The results obtained were such that it will not be necessary to make a distinction between (a) and (b) in discussing the reac-tions. The method which we employed was to take two *Difflugia* of different species and place them near each other in a drop of water, and as soon as one of the animals had extruded a long pseudopod it was cut off and the animal then removed from the drop, leaving its fragment and the other individual behind. At-tempts were made in this way to cross the protoplasm of all the various species of *Difflugia* previously mentioned, viz.: *D. acumi-*

nata, D. corona, D. pyriformis, D. spiralis, and *D. vulgaris.*
While only two or three observations were made on each combination, the results were all negative. Therefore, we feel a degree of assurance in asserting that cross-fusions do not normally take place. The following is one of the most striking observations made on this type of reaction:

On June 10, 1920, a fragment was cut from a *D. spiralis* (animal *A*). This animal was then moved from the field, and a *D. vulgaris* (animal *B*) was brought into contact with the fragment. This contact remained for three minutes, after which animal *B* left the fragment and moved from it to a distance of 200 micra; but within two minutes it had turned around, retraced its course, and made contact with the fragment again. This time such close contact was made that it appeared as though fusion had resulted. Such intimate relations continued for five minutes, then another separation, animal *B* leaving the fragment for the second time. After it had traveled about 300 micra it again turned and came back to the fragment of foreign ectoplasm, making a third contact in about three minutes. This time the two objects did not adhere so closely as on previous occasions. Three minutes later animal *B* moved away from the fragment again, this time remaining away four minutes before returning to make the fourth contact. On this occasion the animal made only slight contact with the fragment, moving off again after a few seconds. When animal *B* had traveled approximately 100 micra, it reversed its course and seemed about to approach the fragment another time. However, before reaching it the animal reversed its course and did not return any more. During the whole procedure (an interval of twenty-six minutes) there was no positive reaction on the part of the fragment of *D. spiralis* toward the *D. vulgaris.*

While it is possible that by using different media different reactions may be obtained, it seems that under conditions comparable to their normal habitat one species of *Difflugia* will not assimilate protoplasm belonging to another species.

VII. Will Individuals Fuse with Protoplasm from Other Individuals of the Same Species?

Being unable to get cross-fusions between different species, we

next sought to determine whether or not one individual would fuse with a pseudopodial fragment from another individual of the same species. In order to make conditions similar to those under which organisms have been repeatedly observed to pick up their own fragments, a portion of the protoplasm was removed from the animal concerned before placing a fragment from another individual near it. The following reaction is an example of what commonly happens when dealing with wild cultures:

Two specimens of *D. spiralis* (*A* and *B*) were placed in the same drop of water, and a fragment was cut from animal *A* at 10:24 A.M., July 14, 1921. This fragment was carried out of the water as it clung to the glass filament with which the cutting was done. Animal *B* was now dragged up to the position shown in drawing (Fig. 8). At 10:29 A.M. fragment *c* was cut from animal *B* without disturbing the position of either cell; the operation did not even cause animal *A* to withdraw its large pseudopod. Specimen *B* was immediately carried from the drop. Animal *A* turned away from the fragment to position *A'* and moved off, but a little later it was moved back to less than half its shell's length from the fragment, only to move away again. For the third time animal *A* was placed near the fragment of foreign ectoplasm, and though one of animal *A*'s pseudopods passed directly over it, there was no reaction either on the part of animal *A* or the fragment from animal *B*.

Other reactions have been observed in which individuals did fuse with fragments from animals of the same species. However, in all reactions of this sort the organisms were obtained from the same culture. There are two possible explanations for this: (*a*) *The individuals were closely related by having a recent common ancestor.* (*b*) *By living in the same surroundings, the environmental influences have acted upon both organisms in such a way as to cause an identical physiological state.* This is left as an open question, upon which further work is being done.

DISCUSSION.

Verworn (1892) cut off pseudopods from *Orbitolites* and observed that after the severed masses had rounded up into little droplets they would adhere to the *ends* of pseudopods extended by

their cell-bodies. Soon thereafter the masses of severed proto-
plasm would bend at the points of contact and the contents would
flow up the pseudopods. Jensen (1896) pointed out that in *Or-
bitolites* and *Amphistegina* chance crossing of pseudopods of dif-
ferent specimens may cause instantaneous shattering of the proto-
plasm concerned, and that the organisms would occasionally pick
up the fragments belonging to themselves and make them again a
part of their bodies. He was also able to get the same results by
cutting off fragments of pseudopods. Furthermore, he observed
that one species would react negatively toward fragments from
other species. Later, in 1901, he made some further experiments
on *Orbitolites* in which he obtained practically the same results.
In the last paper he indicates that there is some response on the
part of the fragments toward restitution. He calls attention to
the fact that tendency toward restitution is stronger just before the
severed fragments lose their power of movement. This does not
correspond to our findings, and since he does not describe his
experiments in detail, it is difficult to know whether or not they
have much in common with those made by us.

There are many unicellular animals that may be cut into two
parts and each part display vital phenomena. In the uninucleated
forms the part, or parts, lacking a nucleus probably carry on only
katabolism or destructive processes. In multinucleated protozoa
we often find each mutilated fragment becoming a new indi-
vidual—as, for example: when a *Stentor, Actinosphærium*, or
Arcella is fragmented. Wilson (1907, '11) separated the cells of
a sponge's body and found that these cells would become organized
again into a new sponge. But this was restitution through segre-
gation and reorganization of parts of a multicellular organism.
In *Difflugia* we have the parts of a cell fusing to retain its former
completeness. In this relation it is impressive to observe how
definitely ectoplasmic fragments respond, in some cases, to their
cell-bodies. As the cell shifts its position the fragment changes
its course correspondingly.

Chemotaxis of some sort is probably involved in the attraction
shown between cell-bodies and their fragments, but that point has
not been determined. Neither mucous trails nor invisible strands
of protoplasm are necessary for the protoplasmic reunion to take

place, for care has been taken to place the animals in such positions that this could be determined. Some substance may emanate from each *Difflugia,* as products of its individual metabolism, which furnishes the required stimulus.

An interesting feature of the reaction between an ectoplasmic fragment and its cell-body is presented by the fact that fusion has never been observed to take place at the tip of an advancing pseudopod, nor at its base, but always along an extended mid-region. This suggests that the ends of such pseudopods must be different from their sides. This, if well founded, carries the analysis of pseudopodial formation a bit beyond that presented by Hyman (1917), who showed that the younger pseudopods of an amœba have a different metabolic gradient from the older ones.

The ability of enucleated fragments to react to stimuli has been observed by others. Hofer (1889) showed that fragments of amœbæ lived for fourteen days; their movements were carried on, though somewhat modified. Verworn (1889) found that enucleated fragments of *Amœba, Difflugia, Lachrymaria, Polystomella,* and *Thalassicolla* live a long time and perform normal movements and normal reactions to stimuli. Minchin (p. 210) makes this statement: " Non-nucleated fragments may continue to live for a certain time; in the case of amœba such fragments may emit pseudopodia, the contractile vacuole continues to pulsate, and acts of ingestion or digestion of food that have begun may continue; but the power of initiating the capture and digestion of food ceases, consequently, all growth is at end, and sooner or later all non-nucleate bodies die off." Lynch (1919) observed that enucleated fragments of an amœba may move, respire, digest, respond to stimuli, and exhibit any activity which is dependent solely upon katabolic or destructive processes of protoplasm. The group of phenomena which they never show constitutes such processes as growth, regeneration, and division. Willis (1916), on the other hand, records that parts without a nucleus do not react as well as nucleated portions—for example: they do not orient with reference to a beam of light. Finally, Mast and Root (1916) say that " the fact that enucleated parts of amœba do not respond at all or respond in a haphazard fashion indicates, as Hofer ('90) concludes, that the nucleus acts as a regulatory center." Hofer,

Willis, and Mast and Root have found, therefore, that enucleated fragments of protozoa do not respond normally, while Verworn and Lynch find that katabolic activity is normal on the part of these enucleated fragments of protoplasm. In *Difflugia* we have found a response that is remarkably well regulated and very delicate. This is of especial interest because the fragments which we were dealing with were only ectoplasmic in composition.

We wish to avoid the implication that the regulated movements of these ectoplasmic fragments were effected without nuclear control, for the absence of nuclei in them does not preclude nuclear influence. Regulatory substances of nuclear origin might have been present in the ectoplasmic fragments at the time they were severed from the cell-body. For, as Minchin (p. 65) states, " in many protozoa, especially amongst the Sarcodina, as, for example, *Arcella, Difflugia,* and many other genera, the cell-body contains, in addition to one or more nuclei, extranuclear granules of chromatin, termed *chromidia,* which may be scattered in the cytoplasm throughout the cell, or may be aggregated in certain regions of the body to form 'chromidial masses' or 'chromidial nets.' "

Regulatory movements between a fragment and a cell were not maintained when a fragment was taken from an individual of one species and placed by the side of an individual of another species. This is in accord with the observations of Wilson (1907), who found that in dealing with dissociated sponge cells, " in contact two masses of the same specific protoplasm tend to fuse," while " unlike specific substances (protoplasms of quite different species) do not tend to fuse." In *Difflugia* protoplasms from two different species do not fuse or coalesce. Also the following quotation from Lillie (1920) can be applied to our findings on *Difflugia:* " If we put two different species of yeast or bacteria into the same culture-medium, each builds up protoplasm of its own kind; *i.e.,* each effects a special predetermined kind of chemical transformation in the materials which it incorporates from the surroundings. Each has the same external materials as its source of supply, but each transforms them in its own specific way, and hence builds up a special kind of protoplasmic structure which, having a special physio-chemical constitution or organization, exhibits corresponding special activities. The term specificity denotes this peculiarity."

This *peculiarity* has been carried a step further in *Difflugia,* for we find that in distantly related individuals of the same species the protoplasm has become so modified in an individualistic way that fusion will not take place between them. Loeb (1920) has recorded observations on the power of tissues to exhibit different reactions to different degrees of family relationship. Our experiments suggest a similar ability on the part of *Difflugia.*

SUMMARY.

1. In *Difflugia* separated pseudopodial fragments are recovered by their cell-bodies, as evidenced by over one hundred experiments. The species included in these observations were: *D. acuminata, D. corona, D. pyriformis, D. spiralis,* and *D. vulgaris.* The distances by which the fragments and their cell-bodies were separated ranged from a few micra to 1,500 micra. The unfavorable conditions under which reunion does not occur are: (*a*) Injury to cell with consequent failure on its part to extrude pseudopods; (*b*) separation from fragment by too great a distance; (*c*) evaporation of water before completion of reactions.

2. The severed fragments are not recovered as food, but enter again immediately into the protoplasmic structure of the cell-body. Such fragments are not reappropriated after they show visible signs of death.

3. In our observations fusion never occurred at the ends of pseudopods, but always took place along an extended mid-region. This indicates a physio-chemical difference between the ends and the middle of such pseudopods.

4. In some cases the severed fragments, which were only ectoplasmic in composition, were observed to take an active part in restitution. Not only did they move toward, but apparently shifted, their line of approach to correspond with the changing positions of their cell-bodies.

5. Fusion has not been observed to take place between two enucleated fragments, even though they be placed in contact.

6. Fusion between an individual of one species and a fragment from an individual of a different species has not been observed. The phenomenon seems to be specific.

7. Observations have been made in which individuals of the same species, obtained from the same wild culture, showed a decidedly negative response toward each other's fragments. Yet in other instances such cross-fusions did occur. This latter suggests the possibility of close genetic origin.

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EXPLANATION OF PLATES.

PLATE I.

FIG. 1. *Difflugia pyriformis.* While at position A a fragment was cut off and left with spherical contour at position w. As cell-body moved to positions B, C, D, E, F, and G, fragment w changed shape and travelled as an ameboid body along path indicated by x, y, and z. z fused with the side of the upper pseudopod of cell-body at G.

FIG. 2. *Diffluga spiralis.* Fragment c cut off at 11:38 A.M. Fragments a and b cut from two pseudopods synchronously at 11:30 A.M. These fragments a, b, c now lay with reference to each other and to the cell-body as indicated. In a little time a slender pseudopod of cell-body came down by side of shell and a was appropriated. The cell-body was now dragged six diameters of its shell to left from b and c. By 11:45 A.M. it had taken up c. It was then disturbed with a glass rod; but at 11:45¾ A.M. it had emerged and taken up fragment b.
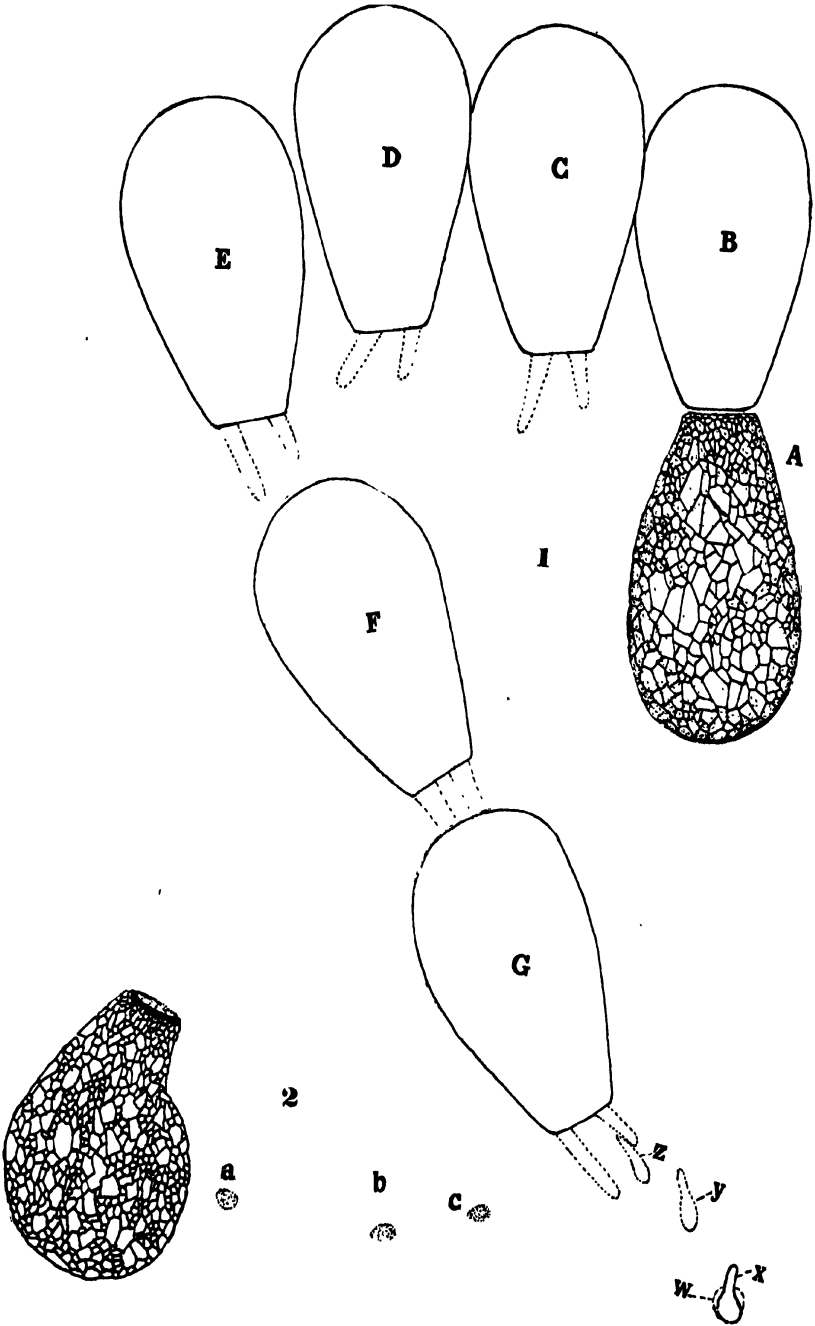
PLATE II.

Fig. 3. *Difflugia spiralis.* *3–A.* Specimen placed in contact with a dead and a nearly dead fragment of its own ectoplasm. The dead fragment was at once rejected, the animal passing it (clinging along upper side of shell at 3-*B*) and dragging the nearly dead (spherical) fragment with it 3-*B*. In time both fragments were rejected.

Fig. 4. *Difflugia pyriformis.* Fragment *a* was whipped off by the sudden retraction of the pseudopod *A*. This fragment lay, of course, in position indicated by end of pseudopod *A*. For graphic reasons it and *b, c, d,* had to be placed below. While fragment *a* lay in this position, it lost its refractive axial rod as in *b;* put out a pseudopod to right as in *c;* assumed contour as in *d*. Next it travelled as an ameboid body to positions *e, f, g*. In the meantime pseudopod *A* had not reappeared and pseudopod *B* was thrown out behind and beyond *g*. When this met *g*, the currents in *g* were reversed and the body of *g* fused with the walls of the cup that *B* had formed about it, as shown at g'; as a result *g* flowed into *B* without leaving any local enlargement of *B*, or being seen as a discrete body within *B*.
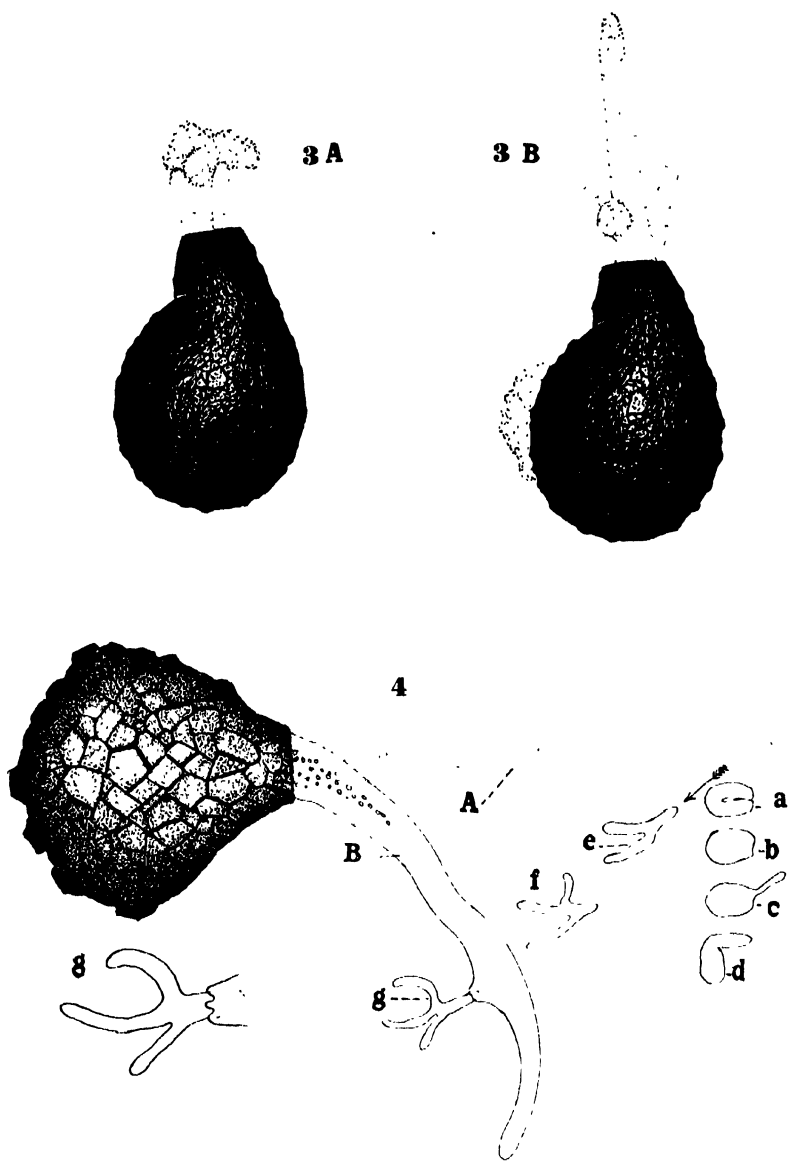
3 A   3 B

4

PLATE III.

FIG. 5. *Difflugia spiralis.* Fragment cut from *a* and dragged to position 1, 1500μ distant, where the fragment had a rod-like contour. Until cell-body had moved through spiral curve along *b*, *c*, and to *d*, the fragment moved about aimlessly through positions 2, 3, 4, 5, 6, 7, 8, (9 the solid black outline not indicated by a numeral). The fragment and cell-body *d* now travelled towards each other, the former along path 10, 11, 12, 13, 14, and 15, the latter coming to position *e* where the two masses of protoplasm met. × 100.

FIG. 5–A. *a* shows fragment, left at 15 in Fig. 5, now travelling down along pseudopod towards a cup that had been formed; *b* union of fragment and cup just completed; *c* and *d* show the details of the end of this process of restitution. × 250.

FIG. 6. *Difflugia pyriformis.* Fragment *b* had moved from position *b* to *x* towards mouth of shell *A*. Cell-body was now moved to position *B* without disturbing either position or contour of *x*. Immediately after *A* was shifted to *B*, *x* changed its course and travelled to *y*. Here, unfortunately, the water had evaporated and in adding more water the fragment was lost. × 250.

FIG. 7. *Difflugia spiralis.* Two ectoplasmic fragments taken from an individual and placed side by side as at *m*. Here both are shown as living ameboid bodies. At *n* they are rounding up indicating the approach of death. While at *o* each fragment has become almost spherical and shows no signs of life. × 250.
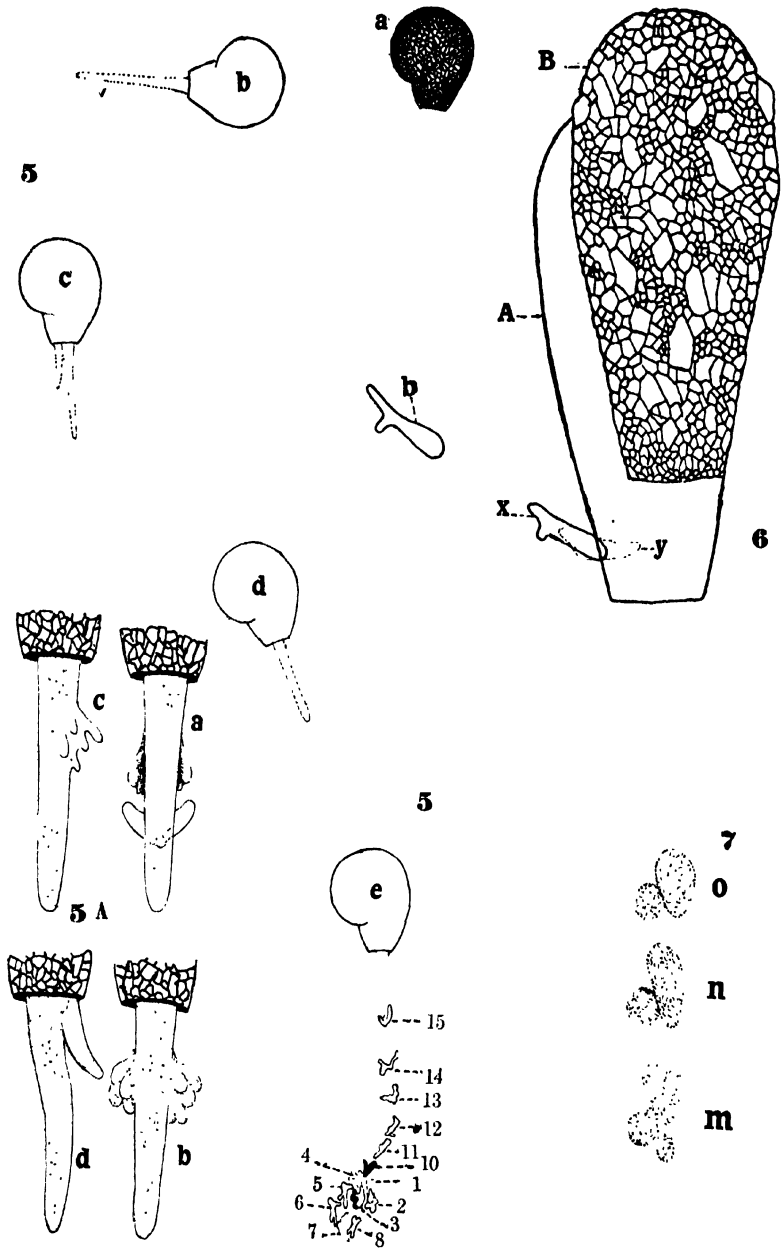
PLATE III.

PLATE IV.

FIG. 8. *Difflugia spiralis*. At 11:29 A.M. specimen *A* lay as indicated in figure. Four minutes earlier a fragment had been taken from a pseudopod of this animal *A* and removed from the drop of water. Specimen *B* was brought to position indicated in figure. Fragment *C* was cut from *B* without disturbing *A* even so much as to cause it to retract its pseudopod. *B* was then removed. *A* travelled to position *A'* and moved out of field. It was then brought back to less than half a shell's length from fragment *C*, but though one of its pseudopods passed directly over *C*, it did not react to fragment *C*, nor did this fragment of *B*'s ectoplasm react towards the incomplete cell *A*. × 250.

PLATE IV.

A'

A

S

B

C
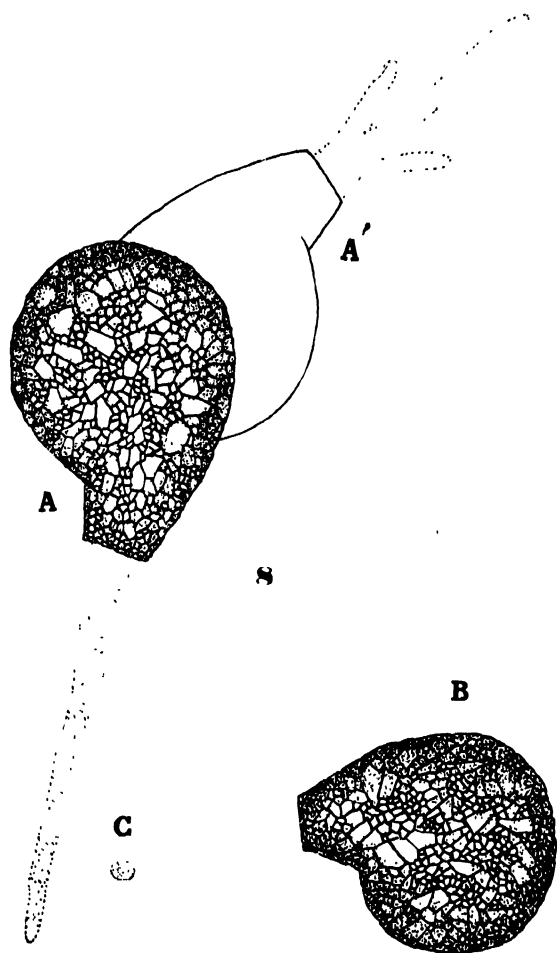
BIOLOGICAL BULLETIN ·

SUPPLEMENTARY NOTES ON TWINS IN CATTLE.

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Since the publication of my previous papers on the free-martin (1916, 1917) more material has been slowly accumulating. The additional data have confirmed and strengthened my former conclusions, and they also include some observations of a certain amount of intrinsic interest. The present paper, therefore, is a series of supplementary notes on twinning in cattle, with comments on more recent literature.

1. CASES WHERE THE FEMALE OF TWO-SEXED PAIRS IS NORMAL.

In my previous paper I concluded that the free-martin is zygotically female, and that its intersexual condition is due to action of the blood of the male twin through anastomosis of the foetal blood vessels which develops very early; Keller and Tandler (1916) came to the same conclusion as the result of entirely independent studies. This theory receives a crucial test in those relatively rare cases of two-sexed twins in which no such anastomosis develops; for in such cases the reproductive system of the female should be normal. In my previous paper I reported three cases of a normal female twinned with a male out of twenty-four pairs of two-sexed twins. The records of the first two cases (Nos. 8 and 9) were, however, incomplete, and the question of vascular anastomosis was not fully investigated because they were received before any theory on the subject was developed. They were, however, consistent with the above interpretation, for it was recorded in the notes made at the time of collection that the connection between the two chorions was narrow; presumably there was no vascular anastomosis. The third case (No. 40) was, however, a perfect test case in every respect; there was a corpus luteum in each

maternal ovary; thé two chorions were entirely separate and there
waš, therefore, no question of vascular anastomosis

Since this publication two additional cases have been received
(Nos. 63 and 64) similar in every respect to case 40   The
chorions were entirely separate in these cases also, and in each pair
both the male and female possessed normal reproductive organs
In a third case, not previously reported (No 93), in which the
female was also normal, the chorions were connected but there was
no vascular union between the two sides   The notebook records
of these cases follow

No 63   December 19 1917   twins in uterus   mothers ovaries missing
Uterus carefully opened   the membranes appear at first sight to be fused
in the body of the uterus   but they were really entirely separate   the
end of one being merely slightly invaginated over the other   so that
they fall apart intact   Length of male and female 2_ cm each   repro
ductive organs of both entirely normal

No 64   February 1 1918   Twins in uterus   both mothers ovaries present
corpus luteum in each   Uterus carefully opened and chorions found to
be entirely separate   In this case the openings from the horns into the
body of the uterus were extremely constricted and the chorions were
confined to the horns   Male 14 cm long   female 13 75 cm long   Re
productive organs of both entirely normal

No 93   December 1 19 0   The twins were in the intact uterus   mothers
ovaries present   a corpus luteum in each ovary   The chorions when
exposed were found to have a very narrow connection   which on ex
amination turned out to be non vascular   Length of male twin 10 25
cm   of female 175 cm   Reproductive organs of both entirely normal

Keller and Tandler (1916) report six cases out of a total of
91 cases of two sexed twins of cattle in which the fœtal membranes
were examined, in which the female was normal   In one of these
the two chorions were adherent but not fused in any way, in
another there was a narrow non vascular strand uniting the two
chorions apparently similar to my case No 93   In the remaining
four cases the chorions were well united but the place of fusion
was indicated by a narrow strip of white, scar like tissue com-
pletely encircling the chorion, in three of these cases no vascular
anastomosis could be demonstrated across the place of fusion; in
the fourth case several exceedingly small blood vessels crossed,
very different from the typical wide connection between the two
circulations   The last case was in the fourth month of pregnancy
(size of fœtuses not given)   The connections in question may

have failed on account of their small size, but it seems more prob-
able that they represented secondary connections, formed too late
to be effective.

We thus have twelve cases on record in which the female of
two-sexed twins of cattle is normal, and in which the conditions
of the foetal membranes are also known—six cases of Keller and
Tandler and six cases of my own. In nine of these cases there
was certainly no vascular connection; two of my own cases were
not satisfactorily investigated with reference to this point, and in
one of Keller and Tandler's cases the fine anastomoses that existed
were apparently secondary. In all other cases of two-sexed calf
twins in which the foetal blood vessels were examined (29 of the
author and 85 of Keller and Tandler) there was anastomosis be-
tween the foetal vessels and the female was definitely intersexual.

The proportion of cases in which the female of two-sexed twins
in cattle is normal was found by Keller and Tandler to be 6 out
of 91; in my collection it is 6 out of 39; Numan estimates the
proportion to be 1 in 8, and Luer (1913) 6 in 113. (Cited from
Keller and Tandler.)

The experiments of Minoura (1921) in which he grafted pieces
of testis or of ovary on the allantoic membrane of chick embryos
and obtained as a result intersexes of various grades, furnish com-
plete proof that sex hormones can produce precisely the same kind
of changes that are found in the free-martin.

2. THE SEX-RATIOS.

The original account involved 54 cases of foetal twin calves
in which the distribution of the sexes was as follows: ♂ ♂ 19,
♂ ♀ 3, ♂ ♀ 21, ♀ ♀ 11; or classifying the normal and intersexual
females of two-sexed pairs together, ♂ ♂ 19, ♂ ♀ 24, ♀ ♀ 11. The
expected ratios, assuming approximate equality of male and female
zygotes in cattle, would be in the proportions of 1 : 2 : 1—*i.e.,* 13.5 :
27 : 13.5. It is obvious that there is an excess of male pairs which
is not readily interpretable as due to chance, even though the num-
bers are relatively small.

I have now 38 additional pairs of foetal twin calves (Nos. 58–97,
excluding No. 66, which was a pair selected after birth, and No. 80,

which was apparently monozygotic) ; the addition of these gives the following distribution of the sexes for all my cases: ♂ ♂ 29, ♂ ♀ 6, ♀ ♂ 33, ♀ ♀ 24; or again combining normal and intersexual females of two-sexed pairs together : ♂ ♂ 29, ♂ ♀ 39, ♀ ♀ 24. Assuming an equality of the sexes in the population dealt with, the expected distribution of sex in the 92 pairs of twins would be ♂ ♂ 23, ♂ ♀ 46, ♀ ♀ 23. It is obvious that there is an excess of male pairs and a deficiency of two-sexed pairs on this assumption. The numbers are not very large, but probably sufficiently so to be significant.

The aberrant ratio for the twins would be satisfactorily explained if the transformation of the free-martin sometimes went so far in the male direction as to render it indistinguishable from a male. I have, therefore, recently studied male pairs with considerable care, but have found no evidences of abnormalities in the reproductive organs; in my experience the structural differences that distinguish a free-martin from a male are always very great. It was natural to expect the greatest modification of the female when both the male and female twin came from the same ovary and developed in the same horn of the uterus, for under these circumstances the vascular anastomosis would presumably be unusually early and extensive. The two cases of this sort which I have, however, show no greater modification of the female than many others (see Sec. 4).

It appeared probable, therefore, that the assumption of approximate numerical equality of the sexes in the population was at fault. I therefore suggested to Mr. J. M. Jewell that he ascertain the sex-ratio in fœtal calves taken from the same slaughter-house. Accordingly the sex-ratio of 1,000 cases taken at random was determined (Jewell, 1921). For our purpose it is sufficient to notice that a sex-ratio of 123.21 was established for the 1,000 cases, including all fœtal ages; the sex-ratio for the sizes included in the twin collection (3.5–30 cm. in length) was found to be 120 (i.e., 120 males for every 100 females, 264 entries).

Taking this figure as the sex-ratio of the population from which the twins came, the expectation of sex distribution in the 92 pairs of twins would be ♂ ♂ 27.37, ♂ ♀ 45.62, ♀ ♀ 19.01. This shows a type of sex distribution similar to that actually found in the twins.

which, however, show a sex-ratio of only 111.5 counted as individuals. The approximation of the twin sex-ratio to the expected sex-ratio is, therefore, sufficiently exact when the smallness of numbers is taken into account to exclude the need of any special assumption.

Incidentally it may be noted what a complete demonstration the figures furnish of the underlying assumption that the free-martin is zygotically female, which D. Berry Hart apparently continues to question (1918). In a recent paper Gowen (1921) presents extensive data showing that twins of cattle, whether of the same sex or both, do not resemble one another in color more than full sisters of the same breed. As the latter are, of course, dizygotic, the presumption is that the twins are so likewise, or at least that monozygotic twins in cattle are extremely rare.

3. On Monozygotic Twinning in Cattle.

The embryological criterion for distinguishing dizygotic and monozygotic twinning in cattle would be the occurrence of two corpora lutea in the maternal ovaries in the former case and of one in the latter. Implantation of the fœtuses in one horn of the uterus would also be expected, but this can not be relied upon by itself as evidence, for one ovary often furnishes two ova, both usually remaining in the horn of the uterus on the same side as the ovary. It is also conceivable that two ova derived one from each ovary might lodge in one horn of the uterus owing to passage of one of them from one horn to the other; however, I have found no such case. The existence of a single chorion would also, of course, be postulated in monozygotic twinning, but its value as evidence would be negligible owing to the tendency for originally separate chorions to fuse in cattle.

In the case of cattle, therefore, the embryological evidence for monozygotic twinning is reduced to the occurrence of only one corpus luteum for two embryos of the same sex. One such case (No. 80) occurs in my collection. The chorion is single, resembling in all respects cases in which the usual two corpora lutea are from one ovary; the amniotic cavities were adjacent, but not communicating; the twins were females of about 16 cm. greatest length. The preparation has been preserved complete in formalin

to demonstrate these points. This may be a case of monozygotic twinning; but the possibility that the Graafian follicle furnishing the corpus luteum contained two ova, both of which were fertilized and developed, can not be neglected.

My records include 36 cases of twins in which both maternal ovaries were present; of these 35 had two corpora lutea either in both ovaries or in one. In the 90 cases reported by Keller and Tandler there were 88 cases of two corpora lutea and 2 of three corpora lutea. Thus only one case of 126 known cases exhibits one corpus luteum for two embryos. These statistics demonstrate how futile it is to continue to appeal to monozygotic twinning, as D. Berry Hart (1918) does, as the cause of the intersexual condition of the free-martin.

4. THE FORMATION OF THE FREE-MARTIN AN "ALL-OR-NONE" REACTION.

Cases of two corpora lutea in one maternal ovary are apparently not very rare in cattle twins, to judge from the statistics of Keller and Tandler, who found 36 such cases out of 90. I have found only three such cases out of 36 cases where both ovaries were present, but the small number may be due to the fact that the butchers, on whom we relied for selecting uteri containing twins, would be likely to overlook twin pregnancies confined to one horn of the uterus. Cases of this kind in which one fœtus is a male and the other a free-martin are of considerable theoretical interest, because implantation of the twins in one horn of the uterus offers presumably the maximum opportunity for modification of the female.

In all cases in which the twins come from two ovaries it is necessary for the chorionic vesicles to grow entirely through their respective horns of the uterus before they can meet in the corpus uteri, and the fusion that takes place there must occur near the apices of the vesicles, as far removed from the embryos as possible. It is probable that the embryos would be about 15 mm. long before fusion (cf. my earlier paper, p. 389), and we have no data for deciding how quickly vascular anastomosis would become established and effective. When, on the other hand, both ova come from one ovary (Fig. 1), and develop in one horn of the uterus,

FIG. 1. Case No. 72. Female twins. To illustrate the membranes when both embryos are derived from one ovary. Two corpora lutea occur in ovary 1, none in ovary 2. Both embryos were in one horn of the uterus. Each 28.5 cm. long. A. P. shows the position of the partition between the two amniotic sacs. An arterial anastomosis is shown in the insert figure which shows the inner surface of the cotyledon at X between the points A and B of the main figure. (× by 5/22.)
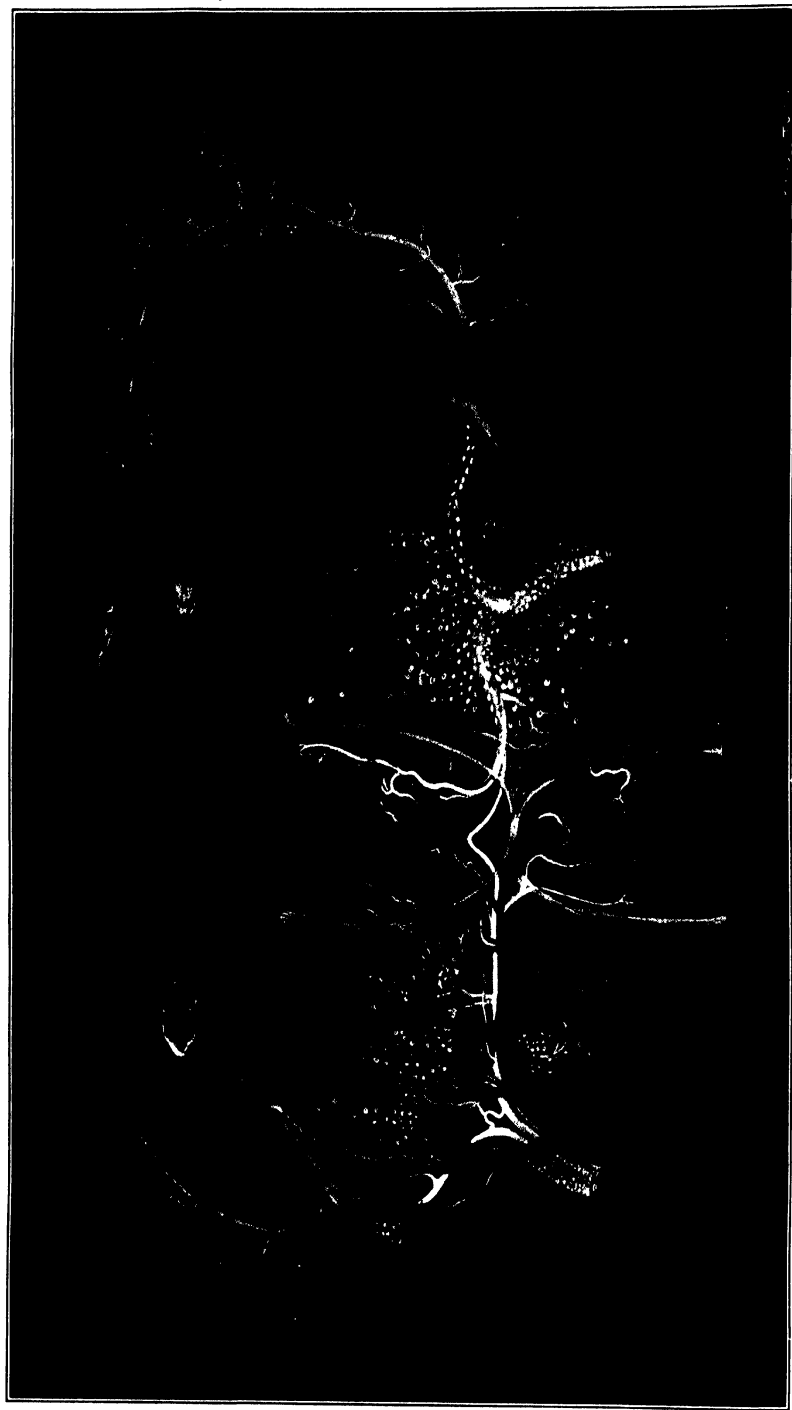
FIG. 2. The membranes of Case 78 described in the text to show the relations of the two amniotic sacs and the strong anastomosis between the umbilical arteries of the twins ($\times$ by about $\frac{1}{4}$). Embryos 32 cm. long

fusion may take place at a much earlier stage; and vascular anasto-
mosis may date from the time of extra-embryonic circulation. The
approximation of the embryos in the common chorion may give
some indications of the manner and time of fusion, and the vascu-
lar relations will also throw some light on the same questions.

I have two cases of this kind (Nos. 78 and 97) in which the
fœtuses are of opposite sex, but in neither case is the modification
of the free-martin particularly extreme. They are so alike that
the description of one of them (No. 78) will suffice. Fig. 2 is
from an oil painting showing the membranes; the very direct
arterial connection between the two umbilical cords will be noted.
The two amniotic cavities are so close together that the walls are
in contact at one place. The fusion of the two original vesicles
was presumably, therefore, side by side and very early, for all
overlapping parts have disappeared without leaving any trace.

If I were to venture to reconstruct the probable history of this
case, I would say that the fusion was probably complete at least
by the 10-mm. stage, and vascular anastomosis established at the
same time. The reasons for this opinion are the extent to which
the membranes of a 10-mm. embryo fill up one horn of the uterus
(see Lillie, 1917, p. 389) and the readiness with which fusion of
the membranes takes place (specifically in cattle) when they are in
contact. If this opinion is correct, the vascular anastomosis in
this case dates from long before the period of beginning of sex
differentiation.

The anatomy of the reproductive organs of the free-martin is
shown in Figs. 3A and 3B. If these figures be compared with
figures of free-martin anatomy given in my 1917 paper and with
Figs. 4 and 5, it will be seen that the extent of the modification is
less than the average in certain respects. Thus the cornua and
corpus uteri are retained in this case, though very rudimentary
compared with the normal; in probably the majority of free-
martins they are entirely absent (cf. Fig. 5); the Wolffian ducts
are also more slender than in most free-martins, and the guber-
nacula less typically developed (cf. Fig. 4). The gonads are ex-
ceptionally minute, which would appear to indicate less than the
average stimulation of the male homologues in the original ovary

out of which the free-martin gonad is entirely composed (cf. Chapin, 1917; Willier, 1921).

Fig. 3. Anatomy of the urinogenital system of the free-martin of Case 78, 32 cm. long. Described in the text. Greatest length, 32 cm. *A*. The entire system. *B*. The urinogenital cord has been cut across in front of the seminal vesicles and turned over anteriorly so as to expose the dorsal surface. 1. The exceedingly minute gonad; 2. Cornua uteri; 3. Gubernacula invaginated into the body cavity; 4. Cut end of umbilical artery; 5. Wolffian ducts; 6. Very slender seminal vesicles. Natural size.

The idea that I have expressed elsewhere (1917) that the variable degree of intersexuality exhibited by free-martins may be a function of the time of onset, intensity, and perhaps duration of
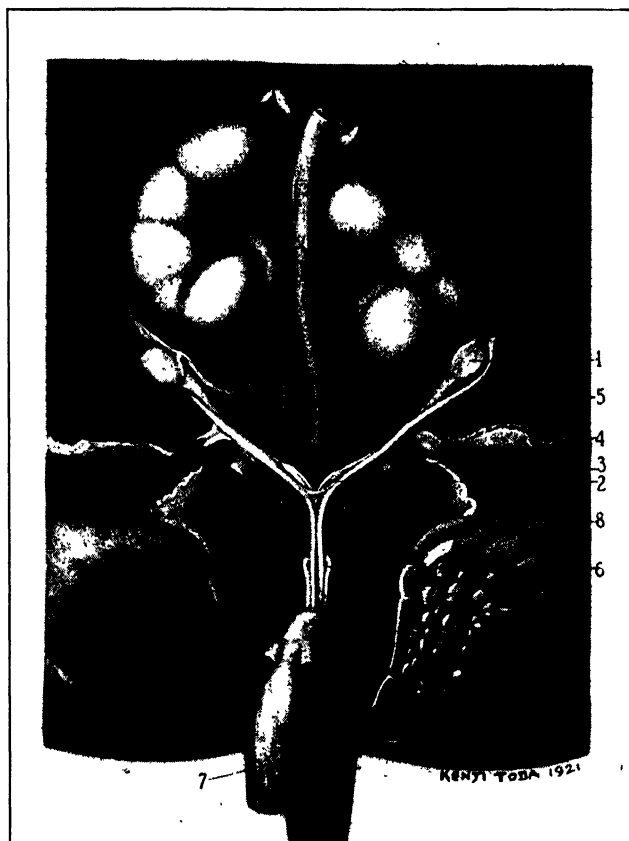
FIG. 4. Anatomy of the urinogenital system of the free-martin of Case 81, 14 cm. long. Described in the text. Greatest length about 14 cm. 1. Gonad; 2. Rudiment of cornua uteri; 3. Gubernaculum; 4. Cut end of umbilical artery; 5. Wolffian duct; 6. Seminal vesicles; 7. Allantoic stalk reflected; 8. Ureter. (× 2.)

action of the male sex hormones requires qualification or sharper definition. The case we are considering would seem to have afforded the maximum opportunity in these respects, without the maximum result following. It should, however, be borne in mind that no one has yet investigated the stages of the free-martin from

32 cm. length to birth (about 100 cm.), and that the fœtal condi-
tions have not, therefore, been adequately correlated with adult
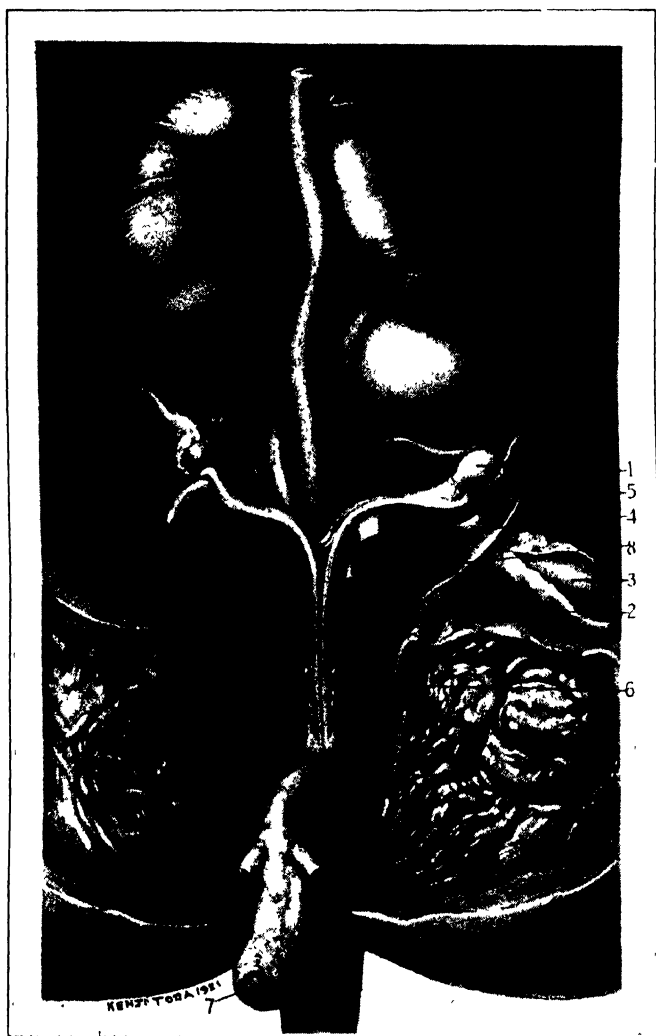
Fig. 5. Anatomy of the urinogenital system of the free-martin of case 94,
19.25 cm. long. Described in text. Greatest length about 19 cm. 1. Gonad;
2. Wolffian duct, 3. Gubernaculum; 4. Cut end of umbilical artery; 5. Wolf-
fian duct; 6. Seminal vesicles; 7. Allantoic stalk reflected; 8. Ureter. (× 2.)

conditions. It would seem that the major portion of the growth
of the gonad of the free-martin occurs in these missing stages, and

it is possible that in the present case the gonad would have grown considerably. But it could not have passed out of the body cavity, for the gubernacula did not enter the body wall in this case. And it is impossible that a male type of external organs could have developed in this case, for the definitive female type was already formed.

Cases at the other extreme from the present one also throw some doubt on the merely quantitative explanation of the degree of intersexuality. I refer to cases where the vascular interconnection is very delicate. One case of this kind, not previously reported, is especially striking (No. 81). In this case I at first doubted the existence of an actual arterial anastomosis, for the injection was not very successful. A careful examination, however, showed an exceedingly delicate artery from the male side passing far into the territory of the female chorion and there apparently anastomosing with an artery from the female. The reproductive system of the free-martin in this case (stage of 14 cm.) was typically intersexual (Fig. 4) and similar in all essential respects to the case shown in Fig. 15 of my former paper: gonads reduced, ducts of the male type with rudiments of the cornua uteri persisting, however, gubernacula present, etc. Indeed, this case is certainly more modified than No. 78.

Keller and Tandler (1916) describe a case in which the vascular arrangements found seemed inadequate to account for the typically deformed condition of the reproductive organs of the free-martin. In this case they state that no strong vascular anastomoses were demonstrable, nevertheless the female exhibited the typical malformation of the reproductive system. They add that a welt-like connection similar to that which usually carries the connecting vessels was found between the main vascular trunks on the two sides, but that it possessed no lumen. They consider this as pointing toward the possibility of an earlier typical connection that became interrupted secondarily.

All free-martins that have been described depart very widely from the female anatomical plan, and also very widely from that of the male. If we were to imagine a sex scale of, say, twenty points, in which points 1, 2, and 3 on the left include the normal female range and 18, 19, and 20 on the right the normal male

range, then I would say, as a matter of quite arbitrary judgment, that the free-martins would occupy points 8 to 13, inclusive, possibly encroaching in some doubtful cases on points 14 and 15. The free-martins, in other words, form an exceedingly well-defined group without intergrades to normal female or normal male. Within the group of free-martins there is considerable range of variation which may be classified according to degree of approximation toward the male type, as B. H. Willier (1920) has shown for the gonads. The ducts also would permit of a similar classification, though it is not certain that the classification by gonads and ducts would always agree.

The absence of intergrades to the female side, and the absence of correlation between the size of the vascular anastomosis and the degree of intersexuality, indicates an "all-or-none" type of reaction in formation of the free-martin; presumably, therefore, the differentiating action concerns a rather definitely delimited period of development and does not require a maximum amount of the male hormones. If this be so, variations of the quantity of the hormones within wide limits would not be significant for the typical reaction. But variation in time of onset with reference to the preceding degree of differentiation of the ovary and other organs of the female may be sufficient to explain in part the range within the free-martin series (cf. Willier, 1920). The matter is discussed further in section 6 (p. 70).

It is interesting to note that Pézard (1920) concludes that the morphogenetic effects of transplanted testicular fragments on castrated cocks also follows an "all-or-none" law; "as soon as the functional threshold is passed, whatever may be the mass of the active gland, a cock takes on as a whole its secondary sexual characteristics. It appears that these manifestations can not be fractionated."

5. On the Occurrence of Seminal Vesicles in the Fœtal Free-Martin.

In my study of the anatomy of the fœtal free-martins (1917) the existence of seminal vesicles in the stages in question was doubted, although they appear in the males in much earlier stages. On the other hand, seminal vesicles appear usually to be present

after birth in free-martins, and I therefore assumed that their appearance in the free-martin was probably belated. A reëxamination of the material, however, shows that they are always present in fœtal free-martins in which the lower part of the Wolffian ducts is developed. Fig. 5 from a specimen hitherto not recorded (case No. 94) shows the typical condition in a free-martin with well-developed Wolffian ducts.

Reëxamination of the specimens figured in my 1917 paper shows similar structures, sometimes more slender, in those represented by Figs. 14, 15, 17, 18, 20, 21, 22, 24, 25, and 27. On the other hand, the specimen shown in Fig. 26 had no Wolffian ducts and no seminal vesicles. The other specimens figured were not available for reëxamination. The specimens were drawn correctly, as dissected at the time the drawings were made; it required further dissection to reveal the seminal vesicles (cf. Fig. 5 of this paper with the figures of the 1917 paper).

This correction removes the necessity for the special assumption made in the 1917 paper that these organs develop out of their due time.

6. THE EARLIEST STAGE OF THE FREE-MARTIN.

The smallest free-martin hitherto described is 7.5 cm. greatest length, case 19 of my 1917 paper. The gross anatomy was described and figured by myself, and the histology by Miss Chapin (1917). The normal female characters are highly modified at this stage; the ovary exhibits a serous covering and albuginea similar to that of the male, absence of the ovarian cortex, and presence of the medullary components of the ovary. In the normal ovary of the same stage the cortex is highly developed, though Miss Chapin states that the cords of Pflüger are not yet separated from the germinal epithelium. The Müllerian ducts of the free-martin have begun to degenerate similar to a normal male of corresponding age (Chapin, p. 459). It is, therefore, apparent that the modifications of the free-martin must start at a considerably earlier stage.

From a study of the chorionic vesicles of normal single pregnancies I concluded that the opportunity for fusion of embryonic membranes from a two-sided twin pregnancy is present from the

10-mm. stage of the embryos, and for vascular anastomosis from the 19-mm. stage, or slightly earlier, because the allantois passes completely through the body of the uterus at that time. This antedates the beginning of visible sex differentiation which I estimated then at about 25 mm., confirmed since by a much more extensive study.

These considerations indicated the possibility of exchange of blood between twin embryos before the beginning of sex differentiation, but it was not demonstrated that this actually occurred, nor yet how early the blood of the male had any sex hormone contained in it.

The case about to be described (No. 62) gives a fairly definitive answer to these problems. The point of chief interest, demonstrated below, is that the hormones in the male blood have produced a practical inversion of the ovary of the female twin at a stage of 3.75 cm., and must hence already have been active for some time. The weight of a male embryo of 3.8 cm. is 3.65 gr.; the weight of its two gonads is 0.0025 gr., or about one fifteen-hundredth that of the embryo. The quantity of interstitial tissue can be only a small fraction of the total mass of the gonads. Nevertheless the secretion of this relatively minute fraction of tissue diluted by the total blood of both embryos is sufficient during growth of about one centimeter in length to reverse the normal course of development of the ovary of the female. I know of nothing that gives a more profound impression of the chemical aspect of development than this demonstrable fact.

Case No. 62: ♂ 4 cm. greatest length, ♀ 3.75 cm.; one in each horn of the uterus; collected in February, 1917. Only one of the maternal ovaries was present and it contained a single corpus luteum. The two chorions were fused so completely that the actual place of fusion can be determined only approximately. Injections of the umbilical arteries with a starch mass did not pass from one side to the other, but at least one perfectly distinct, though very fine, arterial anastomosis by side branches of the main arteries of the two sides could be demonstrated under the binocular.

The two embryos were at first diagnosed doubtfully as males and hence received no further attention for a long time, until I had become familiar with definite external characteristics that en-

abled me to distinguish the sexes with certainty as early as 3 cm.
greatest length.   They were then readily diagnosed as male and
female, respectively, in their external characters.   They were care-
fully studied and compared with normals, with the result that the
reproductive system of the female was found to be already defi-
nitely modified in the characteristics about to be described.

Fig. 6 shows the external organs of the free-martin.   The short

Fig. 6.   Free-martin 3.75 cm. greatest length; Case No. 62.   × 6.   1. Um-
bilical cord; 2. Teats; 3 Phallus.   4. Homologue of scrotal sac of male,
formed in all females; 5. Urinogenital aperture; 6. Anus.

length of the perineum and the development of the urinogenital
aperture are distinctive female characteristics.   In the male the
perineum is several times longer and marked by a ridge; there is
no urinogenital aperture in the male, and the phallus is placed more
anteriorly with reference to the scrotal sacs, which are entirely
similar in the two sexes.

Figs. 7, 8, and 9 show the internal urinogenital organs of the
free-martin (Fig. 7), of its male twin (Fig. 8), and of a normal
female 3.6 cm. greatest length (Fig. 9).   These figures are drawn

with the utmost care as to sizes and proportions of the parts. The much smaller size of the gonads of the free-martin is apparent at a glance. The actual measurements giving the average of the two sides in each case are: free-martin — 1.727 mm. × 0.818 mm.;
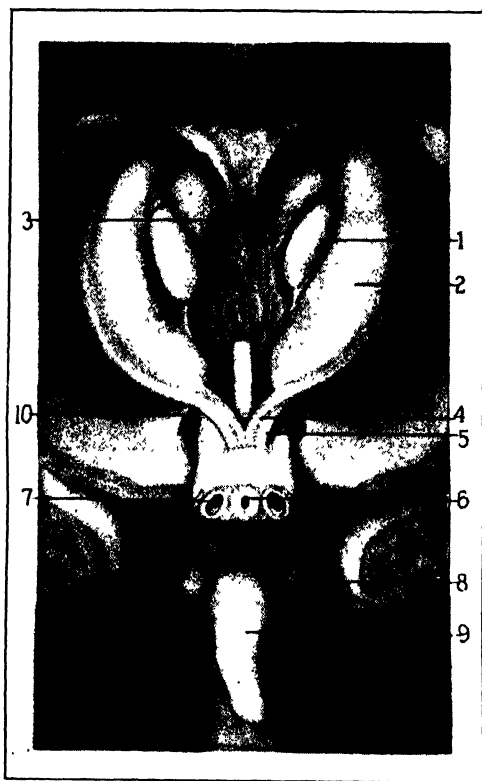
FIG. 7. Internal urinogenital organs of free-martin No. 62 3.75 cm. greatest length, × 6. 1. Gonad; 2. Wolffian body; 3. Vena cava inferior; 4. Müllerian duct; 5. Wolffian duct; 6. Allantois; 7. Umbilical artery; 8. Homologue of scrotal sac of male; 9. Phallus; 10. Inguinal ligament ("round ligament" of uterus).

male twin — 2.622 mm. × 1.346 mm.; normal female — 2.568 mm. × 1.346 mm. The third dimension exhibits at least an equal difference, as may be seen by comparison of the outlines of cross-sections, Fig. 10, a, b, c, drawn at identical magnifications. The male and female normal controls may be regarded as typical for size of the gonads at this stage, for I have examined several other

specimens of each sex between 3 and 4 cm. in length for comparison. The hormone of the male has thus already operated so as to inhibit the growth of the gonad of the female to a very marked extent.

FIG. 8. Internal urinogenital organs of male twin No. 62. 4 cm. greatest length, × 6. 1. Gonad; 2. Wolffian body; 3. Vena cava inferior; 4. Müllerian duct; 5. Wolffian duct; 6. Allantois; 7. Umbilical artery; 8. Scrotal sac; 9. Phallus; 10. Inguinal ligament (part of gubernaculum).

The inhibition in growth is accompanied also by inhibition of the characteristic differentiation of the ovary, though apparently not as yet, to any marked degree, by stimulation of the male homologues in the ovary. In Figs. 11, 12, and 13 comparison is made between the histological structure of segments of those parts of the section outlines of Fig. 10 indicated by the reference num-

bers.   In the male (Fig. 12) the germinal epithelium is already
reduced to a single layer of nuclei, the albuginea has undergone

FIG. 9.   Normal female, 3.6 mm. greatest length (No. 54c) × 6.   1. Gonad;
2. Wolffian body; 3. Vena cava inferior; 4. Müllerian duct; 5. Wolffian duct;
6. Allantois; 7. Umbilical artery; 8. Homologue of scrotal sac of male; 9.
Phallus; 10. Inguinal ligament.

definite fibrous differentiation, and the primary sex cords are form-
ing definite tubule rudiments with abundant interstitial tissue be-

tween. In the female (Fig. 13) the germinal epithelium (about 140 μ in thickness) is in very active proliferation forming several, to many, layers of nuclei. Cords of Pflüger are definitely indicated in the new cortex thus established. The primary albuginea contrasts with that of the male by its more embryonic type. The primary sex cords are not sharply differentiated from the stroma
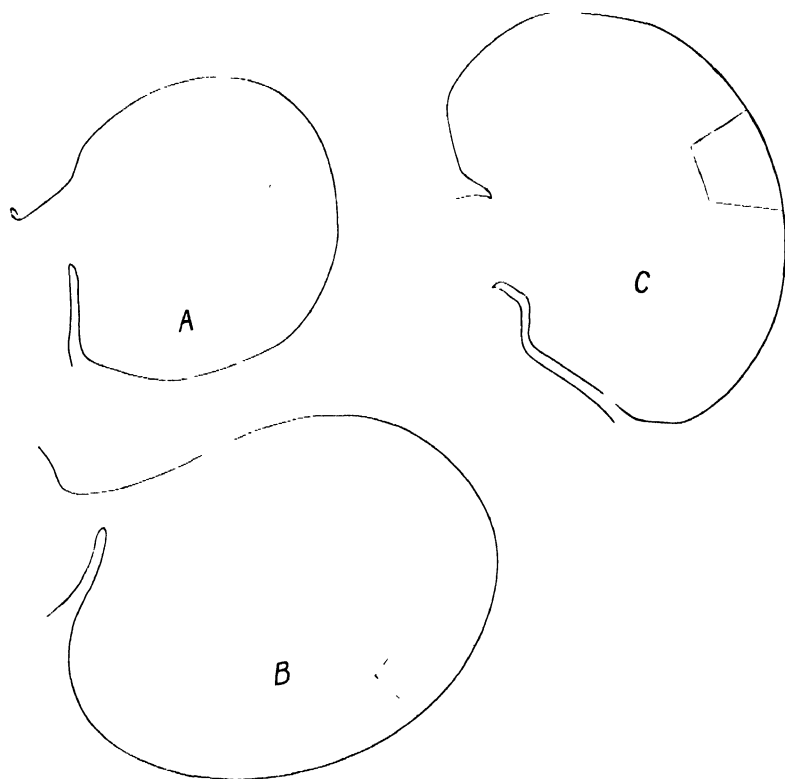
FIG. 10. Outlines of cross sections through center of the gonad, (a) of free-martin No. 62, (b) of male twin No. 62, (c) of normal ♀ No. 54C illustrated in Fig. 9.

of the medulla. The free-martin (Fig. 11) shows the same three layers. The primary albuginea and the medulla do not differ much from those of the female, but the preservation in formalin was not good enough for detailed comparisons on this point. The germinal epithelium is thicker than in the male, but only about one fifth on the average of the thickness of the female. It is, indeed, not as thick as that of a normal female of 3.0 cm. and contrasts

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also with it in the much sparser distribution of the nuclei, evidence
of complete cessation of its activity.

It would thus appear that the characteristic proliferation of the
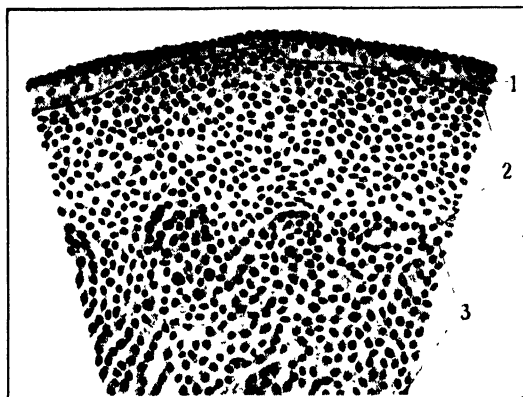
FIG. 11 Detail of area designated in Fig. 10a. Free-martin, No. 62. 1.
Germinal epithelium; 2. Albuginea; 3. Primary sex-cords (medulla).

ovary was halted in the free-martin at a stage not later than 3.0
cm. As will appear from the work of my student, K. F. Bascom,
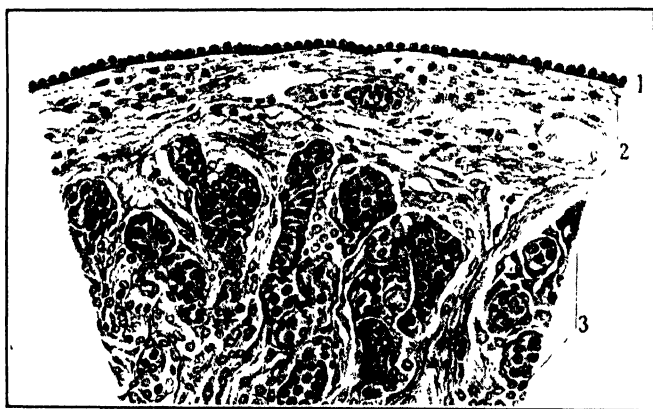
FIG. 12. Detail of area designated in Fig. 10b. Male twin, No. 62. 1.
Germinal epithelium; 2. Albuginea; 3. Primary sex cords (seminiferous tub-
ules) and interstitial tissues.

soon to be published, recognizable differences between the testis
and ovary, concerning the germinal epithelium and albuginea, may

be traced back to the stage of 25 mm. at least. Comparison of these leads to the conviction that in this free-martin ovarian differentiation had begun, and that modification d'd not date from a stage in which the prospective differentiation of the gonad, whether as ovary or testis, is still indistinguishable. However, the work of Chapin (1917) and of Willier (1920) has shown that in later fœtal and post-natal stages nothing remains comparable to the cortex of the ovary.
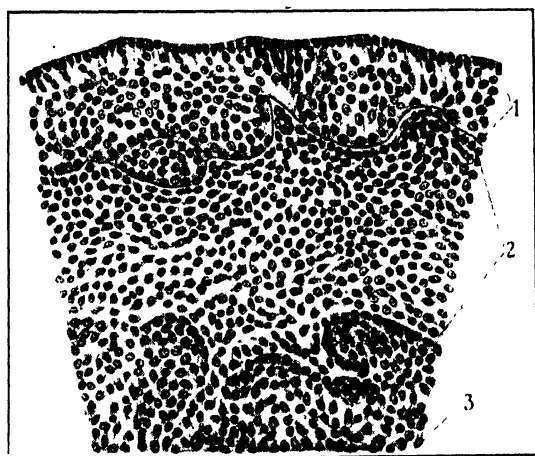
Fig. 13. Detail of area designated in Fig. 10c. Normal female No. 54c. 1. Germinal epithelium (cords of Pflüger) ; 2. Albuginea (primary) ; 3 Primary sex-cords (medulla).

The ostium tubæ abdominale of the normal female is conspicuous at the anterior end of the mesonephros (Fig. 9). It lies in the same position in the normal male (Fig. 8), though it is less conspicuous. In the free-martin (Fig. 7), on the other hand, it can not be seen in the dissection, and was found in sections a considerable distance from the anterior end of the mesonephros, as though the Müllerian duct had failed to grow in length after its formation, and thus its anterior end had become displaced. For the rest there is practically no difference in the ducts in the two sexes; and naturally none was to be expected, nor was any found, in the free-martin.

The feature of most general interest in this case is the demonstration that a specific sex hormone is being produced by the male,

presumable from its testis, at a period very closely approximated
to the beginning of visible sex differentiation, which has a specific
inhibitory action on proliferation of the germinal ep'thelium and
on the rate of growth of the ovary as a whole.

This case is an isolated one so far as the early stage is con-
cerned. There are, therefore, certain restrictions in generalizing
from it. The other evidence that we have demonstrates, however,
that the general relations in this case are typical. But it is prob-
able that actual vascular connection between the two embryos may,
in some cases, be established earlier, and, in other cases, somewhat
later. We considered in section 4 the idea that the quantitative
aspect of the vascular interchange is probably not an important
factor in the degree of intersexuality of the free-martin; that we
are dealing, in other words, with a reaction of the "all-or-none"
type. So far as we can see, then, the principal factor, so far as
hormones are concerned, in determining the range of variation
within the free-martin series is probably the time, in relation to
the early stages of sex differentiation, at which vascular inter-
change is established. But to supplement this, I believe it is neces-
sary to assume that different individuals vary in the state of bal-
ance of the zygotic sex factors, and this may influence the quanti-
tative effect of the hormone factor.

It is probable from these results that the ovary does not produce
a sex hormone at. such early stages. If it were doing so, some
reciprocal effect upon the male ought to be observed in some cases
at least. The histological evidence is just as negative as the physi-
ological in the case of the ovary, while in the case of the testis
fully differentiated interstitial cells are found from the 3 cm. stage
of the embryo on (Lillie and Bascom, '22). Mr. Bascom will deal
with this subject in detail in his study, but I may here call attention
to the fact that all students of ovarian hormones locate the cells of
origin in the cortex of the ovary, and that this part of the ovary
is in its earliest state of origin in the stages in question, and ex-
hibits as yet no differentiation of cells of internal secretion.

Moore (1921) and Sand (1919) have denied Steinach's idea
of a mutual antagonism of testis and ovary of mammals by show-
ing that the coexistence of both gonads in the same body is possi-
ble. The present results show, however, that prior to the forma-

tion of the ovarian hormone the testicular hormone is antagonistic. to growth of the ovary. Presumably, therefore, the normal growth of an ovary in the presence of a testis, as in the experiments of Moore and Sand, depends upon a protective action that the elaborated ovarian hormone exerts against the testicular hormone.   I do not think that Moore (1921, p. 168) is entirely correct in his opinion that hormone action is never characterized by inhibition, and that in the case of the free-martin the transformation of the gonad is due solely to the stimulating action of the male hormone. The gonad of the free-martin is unquestionably inhibited not only in its total growth, but also in its histogenesis by the male hormone; later yet the growth of the Müllerian duct is halted and degeneration also sets in owing to the presence of the male hormone.   It is not until a still later period that the stimulating effects of the male hormone on the sex cords and rete of the gonad and on the epididymis and Wolffian duct of the free-martin become apparent.   There is certainly an element of truth in the contention of Steinach, Lipschütz, and others that the sex hormones inhibit heterologous sex characters in addition to stimulating the homologous ones.

The great and important advances made in recent years in the study of sex hormones have led certain authors to extreme positions concerning their economy in the organism. Lipschütz (1919, p. 390 ff.) has given the boldest statement in his theory that the embryonic soma is primarily asexual, and that sex characters are secondarily imposed upon it by the differentiation of a male or female "puberty gland." In the discussion of this statement we should no doubt run foul of definitions. Whether it is to be understood under the presupposition of the zygotic *determination* of sex or not is not clear. If so, it confines itself to the phenomena of sex *differentiation*. The primary differentiation, therefore, which itself requires explanation, would be presumably that of the misnamed "puberty gland." This is, however, demonstrably not the case (Lillie and Bascom, '22). Moreover, if there were no other factor at work in determining the sex differentiation of embryonic primordia than the specific sex hormone, it is difficult to understand why the free-martin, which receives only male sex hormones, should not become completely male. It is obvious that the male hormone

is acting against resistance in the female soma; moreover, this resistance is not that of already differentiated parts, for the hormone is introduced before sexual differentiation; it is rather a constitutional resistance native to the determined sex. The phenomena can be understood only on the assumption that the zygotic sex-determining factors are also sex-differentiating factors in mammals as in insects. These factors are reinforced very early by hormone production in the male of mammals, but relatively very late in the female. Such reinforcement can be thought of in the sense of an autocatalytic reaction (as suggested by certain writers, cf. Goldschmidt, R., Huxley, J., 1920), in which the hormones are regarded as by-products of the sex-determining factors present in the zygote, that accelerate the progress of the differentiation.

GENERAL DISCUSSION.

On account of certain misunderstandings that appear in the literature since publication of my 1917 paper, I would like to point out the limitations of the theory of the free-martin. I have so far confined the explanation to the bovine free-martin, though indicating the possibility that it may have a wider application. Minoura's experiments (1921) confirm the general applicability of the principle. The explanation is not supposed, however, to apply to all cases of hermaphroditism in cattle; it is not only possible, but probable, that certain cases rest upon a zygotic basis, for hermaphroditism is reported for single births in some cases. The possibility, therefore, exists of an accidental combination of such a case with twinning, though it would necessarily be very rare. There is, however, a sufficient basis for receiving with a certain amount of skepticism cases alleged to be due to twinning in which the history is based on statements of farmers, and not on the personal observations of the investigator. Most of the cases in the literature are, as a matter of fact, of this nature. There is no good reason for rejecting them so long as they are conformable to cases known to be due to twinning; but when they are quite unconformable, especially if they involve extensive modifications of the external parts, judgment may be reserved until proof is forthcoming that such modifications may be due to twinning.

I regard the explanation of the bovine free-martin which I have

given as proved by the large body of evidence accumulated by Keller and Tandler and by myself. Esther Rickards and F. Wood Jones (1918) find unconformable cases in goats described by themselves and others and conclude that these "show the unsoundness of Lillie's theories": A pair of apparently female goat kids turn out to be quite identically predominantly male in their anatomy; "it is affirmed of a celebrated he-goat 'that one season *every* kid he sired, eleven in number, was a hermaphrodite'" (quoted from Davies, 1913). Such cases imply a genetic foundation which certainly deserves study and promises interesting results, but they obviously do not have any bearing at all on the bovine free-martin.

Keller and Tandler (1916) investigated the membranes in four cases of triplets and two cases of twins in goats. In two of the triplet cases three corpora lutea were found in each case, and in one of the twin cases there were two corpora lutea. The ovaries were not available in the other two cases. The chorions were fused, but the individual fœtal circulations were separate, as in sheep (cf. Lillie, 1917). The fœtuses of four of these cases, which included both sexes, were examined and found normal in the anatomy of their reproductive organs. These findings thus confirm, as far as they go, the idea that hermaphrodites in goats are not usually a result of twinning as such; but they do not exclude the possibility of the occasional occurrence of intersexual conditions due to hormones. It is obvious that goats are not favorable material for the investigation of the action of sex hormones in fœtal life.

Hartman (1920) suggests that certain cases of hermaphroditism in mammals may be interpreted as "reciprocals" of the free-martin—that is, cases in which the male of a two-sexed pair is modified in its sexual development by hormones of the female. The evidence that he offers is of the slenderest description. Moreover, if, as seems probable, the female in mammals does not produce sex hormones during the early part of fœtal life, the possibility of "reciprocal" free-martins would seem to be excluded. The source of the ovarian sex hormone is by no means so certainly ascertained as that of the testicular hormone. The weight of opinion inclines to the view that the cells of origin in the ovary are

derived from atretic follicles. If this view should be confirmed, the time of first formation of specific female sex hormones would be postponed in most species until after birth.

Doncaster's suggestion (1920) that the tortoise-shell tomcat may be a female transformed by hormones of a male partner in foetal life has been dealt with by C. C. Little (1920, 1921), who finds no vascular connections between the membranes of foetal cats. The suggestion, however, leads me to emphasize the finding that conditions in the free-martin, which offer probably the most favorable possible opportunity, do not lend themselves to the idea that complete sex inversion by hormones is a possible thing. I am led to emphasize this because Julian Huxley (1922, pp. 201 and 214) also states, basing his statement on the free-martin, that hormones may produce all stages of conversion of one sex into the other. Although I have paid particular attention to this point, I have been able to find no evidence for it. On the contrary, the conversion of the female sex in the case of the free-martin stops very far short of complete inversion. There is a wide gap between the most extreme case and the normal male, and the statistics do not favor the suggestion that in some cases this gap may be crossed at a bound, leaving no intermediates (cf. p. 50). I believe, however, that proper caution admonishes us to realize that the limits of hormone action may vary, even widely, in different species. Both Steinach and Moore, for instance, find considerable differences between the rat and guinea-pig in the feminizing effect of ovarian grafts on castrated infantile males. I do not, therefore, mean to deny the possibility of complete sex inversion by hormones for all species, but merely to emphasize that the case is not proved for any species.

So far it has not been shown that the explanation of the intersexual condition in the free-martin is of wider application for the explanation of intersexuality in mammals. The theories must, however, develop along two main lines, viz.: either that of unbalanced sex factors or of unbalanced hormones. The former is a problem in genetics, which will be advanced by actual breeding experiments, for which goats seem to offer unusual advantages owing to the relative abundance of hermaphrodites in certain breeds, and the negative evidence against the theory of unbalanced

sex hormones in this case. The sex hormones may be unbalanced as against each other or as against genetic sex factors in other ways than by anastomosis of fœtal circulations in different-sexed individuals. As I pointed out previously, there may be a breaking down in the presumable defence mechanism of the placenta that protects the embryos from the sex hormones of the mother. Possibly other causes may also exist which in the present state of development of sex hormone theory can not be anticipated.

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Appendix.

Supplementary List of Cattle Twins Examined in Utero.

The list published previously included the first 57 cases. A comparable table of cases 58–92 is herewith given to make the record complete.

SUPPLEMENTARY LIST OF CATTLE TWINS EXAMINED IN UTERO.

No.	Sex.			Size.	Chorion.	Maternal Ovaries.
	♂	♀	☿			
58	2			14.5 cm. each.	Single.	Ovaries absent.
59		2		19.5 cm. each.	Single.	Ovaries absent.
60	1		1	♂ 16 cm., ☿ 15.2 cm.	Single.	One ovary present with corpus luteum.
61		2		23 cm., 24 cm.	Single.	Ovaries absent.
62	1		1	♂ 4 cm., ☿ 3.75cm.	Single.	One ovary present with corpus luteum.
63	1	1		22 cm. each.	Chorions separate.	Ovaries absent.
64	1	1		♂ 14 cm., ♀ 13.75 cm.	Chorions separate.	Both ovaries present. Corpus lut. in each.
65	1		1	♂ 17 cm., ☿ 16.75 cm.	Single.	Both ovaries present. Corpus lut. in each.
66	See Note 1.					
67		2		10 cm., 10.25 cm.	Single.	Ovaries absent.
68	2			9.5 cm., 10.3 cm.	Single.	Ovaries absent.
69	2			7.8 cm., 8.2 cm.	Single.	Both ovaries present. Corpus lut. in each.
70	2			13 cm. each.	Single.	One ovary present with corpus luteum.
71	2			17 cm., 18 cm.	Single. Narrow connection.	Both ovaries present. Corpus lut. in each.
72		2		28.5 cm. each.	Single. Both embryos in one horn of uterus.	Both ovaries present. 2 c. l. in one. None in other.
73	2			6 cm. each.	Single.	Both ovaries present. Corpus lut. in each.
74		2		12.5 cm., 13.5 cm.	Single.	Ovaries absent.
75	1		1	16 cm. each.	Single.	Ovaries absent.
76		2		15.5 cm., 16 cm.	Single.	Both ovaries present. Corpus lut. in each.
77	1		1	♂ 10 cm., ☿ 9.4 cm.	Single.	Ovaries absent.
78	1		1	32 cm. each.	Single. Both embryos in one horn of uterus	Both ovaries present. Two corpora lutea in one. None in other.

Note 1.—Five days' old free-martin, twin to bull. Preserved material for histology. See Willier ('20).

No.	Sex. ♂	♀	☿	Size.	Chorion.	Maternal Ovaries.
79		2		26 cm., 27 cm.	Single.	One ovary present with corpus luteum.
80		2		16 cm. each.	Single. Both embryos in one horn of uterus.	Both ovaries present but only one corpus luteum.
81	1		1	14 cm. each.	Single. Narrow connection.	Ovaries absent.
82	2			13 cm. each.	Single.	One ovary present with corpus luteum.
83		2		12 cm. each.	Single.	Both ovaries present Corpus lut. in each.
84		2		15 cm. each.	Single.	Both ovaries present Corpus lut. in each.
85	1		1	11.5 cm. each.	Single.	One ovary present with corpus luteum.
86		2				
87		2				
88		2		Note 2.		
89	2					
90	2					
91	1		1	15 cm. each.	Single.	Ovaries absent
92	1		1	♂ 12 cm , ☿ 11 5 cm.	Single.	Ovaries absent.
93	1	1		♂ 10.25 cm , ♀ 7.75 cm.	Narrow non-vascular connection.	Both ovaries present Corpus lut. in each.
94	1		1	♂ 19 25 cm., ☿ 19 (?) cm.	Single.	Both ovaries present, Corpus lut. in each.
95	2			4 and 5 cm.	Single without constriction. Both embryos in one horn.	Both ovaries present, two corpora lutea in one; none in the other.
96		2		Not recorded.		Absent
97	1		1	About 15 cm. each.	Single, without constriction. Both embryos in one horn.	Absent.

Note 2.—Numbers 86 to 90 inclusive were collected during my absence in January and February, 1920; no records but sex were taken

COPPER, ENZYMES, AND FERTILIZATION.

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I. INTRODUCTION.

The inhibition of development in *Arbacia* by infinitesimal quantities of copper salts has lately been employed by F. R. Lillie ($21^{1, 2}$) as an expedient in studying the fertilization reaction. The results are highly suggestive and the novelty of the procedure altogether likely to inspire much further work. Yet even now there are complications to be dealt with before one can realize on the possibilities which apparently inhere in the method.

In the first place, there is the matter of effective concentration. This has two aspects: how much of the copper salt added to sea-water actually remains in solution and what proportion of the permanently soluble fraction reaches the eggs? Moreover, is the chorion involved? There is no reason for thinking that copper would escape concentration in the egg-jelly since, as I have recently shown (22^{1}) this is true of all the salts to which the egg is normally exposed. And, finally, is it possible for a marine organism to carry a "copper-avid substance in the cortex of the egg" and escape the all but inevitable consequence?

II. THE PRECIPITATION OF COPPER SULPHATE IN SEA-WATER.

The first step was a study of the solubility of copper sulphate in sea-water. Since my program involved reliance on chemical, rather than physiological, means of detection, I employed concentrations well within the sensitivity range of my first methods. I therefore prepared a standard solution of $CuSO_4 . 5H_2O$ in distilled water such that each cubic centimeter corresponded to one milligram of the original salt. This was equivalent to .276 mg. of metallic copper per c.c., or $Cu = m/230$ and $\#/460$.

This standard was compared colorimetrically with a duplicate solution in sea-water. After correcting for the few drops of ammonia added in order to intensify the blue color, it was found

in three closely concordant experiments that 36.9 per cent. of the copper had been precipitated in the sea-water—in all probability— as insoluble basic carbonates. The sea-water, therefore, retained in solution a trifle over .174 mg. Cu per c.c.—the equivalent of $Cu = m/365$ or $n/730$.

III. THE REMOVAL OF COPPER FROM SOLUTION BY NORMAL EGGS.

The precipitated fraction of the copper sulphate interferes seriously with attempts to measure colorimetrically the quantities actually removed by the eggs. Not only is the turbidity of the solution objectionable, but the precipitate adheres mechanically to the chorion, which in turn goes slowly into solution. It is absolutely essential to eliminate the copper carbonates.   .

However, a concentration of $n/730$, even after the carbonates are removed, often destroys the eggs in all except brief exposures. This difficulty can be overcome by dilution to $n/1,460$. At this concentration .2 c.c. of dry ripe eggs in 75 minutes reduced the copper content of 14.8 c.c. of solution as follows:

Lot A from $n/1,460$ to $n/1,810.4$.
Lot B from $n/1,460$ to $n/1,766.6$.
Lot C from $n/1,460$ to $n/1,785.5$.

Very definitely, then, *Arbacia* eggs are able to remove copper from solution, yet, despite their close agreement, the figures in reality have no quantitative standing. They completely mask an important source of error.

IV. THE RÔLE OF THE CHORION.

If the chorion takes part in lowering the concentration of copper solutions, eggs without jelly should lose at least part of their effectiveness. But to demonstrate this is not easy. The very removal of the chorion, of itself, introduces variables which, in the present case at least, might be increased in number or aggravated by the use of dilute hydrochloric acid as a solvent of the jelly. This restricts us to the mechanical methods with their relative violence and uncertainty. One can never feel confident that every egg has lost its chorion, nor that the shaking, flipping—or whatever one does—leaves every egg intact. Some control of these errors

is possible by microscopic examination in suspensions of india ink, and, as a matter of fact, I never employed material unless the eggs appeared uninjured, nor unless at least 90 per cent. failed to reveal the jelly. However, it was impossible to offset the fact that equal volumes of normal and dechorionized eggs can not contain equal numbers. It is not surprising, therefore, that comparisons with normal material should prove variable; nevertheless, in view of my other experiments, I did not expect to encounter the irregularities actually found. These were so great that the results are best appreciated in the following form:

	Excess Copper Removed in 75 minutes from 10 cc. n/1460 Solution by .2 cc. Normal Eggs	.2 cc. Dechorionized Eggs
Lot A	23.3%	
Lot B	.	11.5%
Lot C	29.5%	
Lot D	4 0%	
Lot E		2.0%
Lot F		8.3%
Lot G	13.7%	
Lot H	54.0%	
Ratio	5.7	: 1

This clearly does not indicate that dechorionized eggs remove no copper from solution, but that normal eggs, on the average, remove more. If we neglect the numerical differences in equal volumes of the two classes of eggs—a handicap distinctly against the normal material—we can say that the copper profit of normal was to that of dechorionized eggs at least as 5.7 : 1.

It follows that the chorion is heavily involved in the removal of copper from solution. Yet the amounts taken up can not be determined even from my original values; nor can these be used as a basis for predicting the precise outcome of any particular competition for copper between normal and dechorionized eggs. Indeed, the results, though valid enough, once more mask a fundamental source of error.

V. Irregularities in the Removal of Copper from Solution.

The irregularities encountered were of several sorts. Equal quantities of eggs, during equal exposures, did not invariably ap-

pear to remove equal quantities of copper from a $n/1,460$ solution. In fact, in certain instances, the solutions after exposure to the eggs seemed to contain more copper than I had added. With dechorionized material the results were almost bizarre. Such eggs usually appeared to absorb less than the normal, though at times the opposite was true. Their most frequent irregularity was the apparent addition of copper to the sea-water. Where was the error?

I sharpened my technique as much as possible. After each experiment a few drops of sulphuric acid were added to the solution to be tested in order that any copper carbonates formed during the experiments might be converted. I also used ammonia to intensify the colors. Nevertheless the harvest of inconsistencies continued.

It was then that I recalled Lillie's " copper-avid substance in the cortex of the egg." This might explain everything, yet only if it could be shown that the untreated normal *Arbacia* egg actually contains copper.

VI.   DEMONSTRATION OF COPPER IN *Arbacia* EGGS.

A.   Preliminary Considerations.

· Was there any likelihood of demonstrating copper in the normal egg? In the literature I found the following especially pertinent facts:

100 gr. fresh *Asterias rubens* contain 2.45 mg. Cu.

100 gr. fresh *Stichopus regalis* contain 2.83 mg. Cu.

100 Sepia eggs contain 0.5–0.8 mg. Cu.

100 gr. dry hen's yolk contain 2.00 mg. Cu.

The first three items are from Aron's ('09) discussion in Oppenheimer's Handbuch; the last from an investigation by Fleurent and Levi ('10[1]).

Measurable quantities of copper, then, have been reported for certain echinoderms and at least two kinds of .eggs.

B.   Qualitative Tests for Copper.

It is hardly necessary to discuss the very large number of preliminary tests made at Woods Hole during the summer of 1921.

It is sufficient to say that whenever I was able to avoid interference, due to small amounts of iron, I found positive indications for traces of copper. Material was, therefore, prepared for the more careful analyses made this winter at Amherst.

Immature eggs were taken directly from the ovaries; mature eggs were shed spontaneously under conditions which I have described elsewhere (22[1]) and which precluded any contact whatever with sea-water, fresh water, dermal secretions, appendages, or detritus of any kind. The eggs were tested with sperm and found to be normal.

Two c.c. of mature eggs dissolved in concentrated nitric acid constitute sample A. This was subsequently diluted to 50 c.c. with water doubly distilled from glass. Fractions of this material were used either directly or, after evaporation to dryness and incineration in glazed china crucibles. The ash was redissolved in sulphuric acid, usually neutralized with ammonia, and the volume restored to that of the original fraction. In all these operations great care is essential. All reagents were tested repeatedly and found negative for copper.

1. *Copper as Cupric Hydroxide*—$Cu(OH)_2$.

   1 c.c. of A evaporated to dryness and incinerated. Ash dissolved in H_2SO_4. Excess NH_4OH should give the blue of cupric hydroxide. Result: positive but exceedingly faint.

2. *Copper as Cupric Cyanide*—$Cu(CN)_2$.

   To 5 c c. of A, added concentrated KCN solution. Should give yellow precipitate of $Cu(CN)_2$. The precipitate should dissolve in H_2SO_4 and this solution should give test for $Cu(OH)_2$. Result: positive and distinct throughout.

3. *Copper as Cupric Ferrocyanide*—$Cu_2Fe(CN)_6$.

   Ash from 10 c.c. of A redissolved and neutralized as before. To avoid interference by iron, the reagent, a 1:35 solution of potassium ferrocyanide, $K_4Fe(CN)_6$ in distilled water, was carefully run over the surface of the test solution as in the ring reaction for proteins with nitric acid. In the present case also a ring is formed; this quickly thickens—the upper layers being composed of the blue ferrocyanide of iron; the lower of brown cupric ferrocyanide.

4. *Copper as Cupric Xanthate*—$Cu(C_3H_5S_2O)_2$.

   Redissolved ash from A, in the presence of potassium ethyl xanthate should give a yellow color due to the formation of copper xanthate

The test which is sensitive roughly to 1 part in 50 million, is not vitiated by the presence of sea-salts nor by small amounts of iron, lead, nickel, cobalt, zinc, or manganese. Results: Positive and very decided.

5. *Copper as Crystalline Cupric Sulphate*—$CuSO_4 \cdot 5H_2O$.

After precipitation of the iron as hydroxide the ash was redissolved in sulphuric acid and drops of the solution allowed to evaporate to dryness on a slide. Under the microscope long blue needles corresponding to copper sulphate. The blue color greatly intensified by ammonia.

6. *Copper as Metallic Copper*—Cu.

5 c.c. of *A* plus excess NH_4OH. Copper if present in sufficient quantity should be deposited on tin-foil. Result: positive but faint With aluminum, however, despite incompleteness, the deposition was very marked; film, rose-colored by reflected light; bright and typically copper-colored when polished.

7. *Copper by Electrolytic Deposition*.

10 c.c. of *A*. Platinum Electrodes. Voltage 2.6; amperage 1/10. At this tension copper and only copper can be deposited on the kathode. After 24 hours, result positive and distinct. Could not be weighed. Redissolved deposit and got positive test with potassium ethyl xanthate

On the basis of this evidence it was no longer possible to doubt the existence of copper in the normal *Arbacia* egg. The basic necessity for a secretion of copper compounds or copper-bearing substances is therefore given. If such materials are held up tem-porarily by the chorion, the differences between normal and de-chorionized ova, noted in sections IV. and V., would be explained. But do the eggs really secrete copper in any form? We could perhaps answer this question if we could associate the metal with definite structures and substances in the egg.

VII. LOCALIZATION OF COPPER IN THE *Arbacia* EGG.

The problem calls for the localization of the copper. I em-ployed direct methods as well as indirect. The eggs used were shed under the conditions previously mentioned and fixed in abso-lute alcohol. I studied both whole mounts and sections, 5μ in thickness. The latter, after removal of the paraffin, like the un-sectioned eggs, were treated with various reagents and examined in the opaque condition, wet or dry; or after clearing, as trans-parent objects, in glycerine or balsam. All the reagents were tested and again found negative for copper.

1. *Copper in the Egg Pigment.*

Since copper is an integral part of so many organic coloring matters, it was natural to examine first of all the pigment of the egg. This material, as is well known, is widely distributed in granular form in the cytoplasm. As the granules are most numerous in tangential sections, the pigment bodies are evidently concentrated near the surface. This also seems to be true, to some extent, immediately about the nucleus.

It is entirely justifiable to identify these granules as the source of the coloring matter secreted by the eggs. I consequently precipitated the pigment from normal egg secretion by the chloroform method described in an earlier paper (21[3]). After incineration an alkaline solution of the ash in twenty minutes gave a relatively heavy, though incomplete, deposition of metallic copper on aluminum. The deposit was dissolved and gave a deep yellow color on addition of potassium ethyl xanthate.

Direct proof of copper in the pigment bodies *in situ* is also possible. Tangential sections which contain more of these granules than others are the very ones that stain most heavily with hæmatoxylin. This we should expect in the presence of copper, though the test is not dependable if iron is also present. I therefore adopted a procedure employed in the microchemical analysis of minerals. The method depends on the formation of a triple nitrite of potassium, copper, and lead ('94).

To sections on the slide I added a trace of sodium acetate and a somewhat larger quantity of potassium nitrite. The whole was then acidulated with acetic acid. Last of all, a few grains of lead acetate. The presence of copper, as little as 0.05 microgram,[1] if crystals are desired, completes the conditions necessary for the formation of the triple salt, $K_2CuPb(NO_2)_6$, which is jet black.

In this test, which differentiates between iron and copper, the sections most darkened are the very kind most affected by hæmatoxylin. There is a marked graying of the cell contents to which a partial reduction of the pigment bodies contributes. The general effect is due to a decided increase in cytoplasmic granulation—the granules being excessively minute and, like the microsomes of

[1] 1 microgram (μgr.) = 1/1000th milligram.

the cytologists, black.   If the material is kept moderately warm on a water bath and the reaction is permitted to continue for half an hour, the pigment bodies themselves become distinctly bluish or even black.   This suggests a gradual unmasking of the copper in Macallum's sense ('12).

2. *Copper in the Egg Membrane.*

A definite visible membrane ('13[1]) invests the unfertilized *Arbacia* egg and constitutes a barrier through which pigment must pass in order to reach the outside.   If now the pigment itself contains copper, the same thing might easily be true of the membrane through which it passes.

But the membrane is by nature yellowish-brown.   This excludes tests depending on the formation of cupric cyanide, ferrocyanide, or xanthate.   With hæmatoxylin a bluish tint develops, and this could be interpreted as evidence of copper if iron were absent. However, since the latter is almost certainly present, we must fall back on the triple-nitrite reaction.

In this the original native yellow-brown of the membrane is replaced with black.   Under low powers the membranes are in very sharp relief.   The oil immersion, however, resolves their uniform black, in optical section, into a series of irregularly spaced discontinuous beads connected by an exceedingly thin continuous black line.

This test was repeated on several sets of sections.   The results were uniform and without inconsistencies.   The evidence, then, that copper exists in the *Arbacia* egg membrane appears valid; yet the quantities involved are so small that I felt impelled to check the results on the hen's egg, whose vitelline membrane, I reasoned, should contain copper if the analysis of the yolk by Fleurent and Levi (*loc. cit.*) is correct.

This supposition was readily substantiated by four different methods.

The vitelline membranes were removed from the yolk and washed in a stream of running tap-water for twelve hours.   In this way all but negligible quantities of yolk were removed.

After incineration a solution of the ash, upon the addition of ammonia, turned blue.   This test was supported in neutral solu-

tion by a marked yellow brought about by potassium ethyl xanthate. Again the triple-nitrite test was positive. Under the microscope the treated membranes showed numerous irregular blotches and specks of black. By prolonging this treatment for thirty minutes and following it with a short exposure to 100° C., the membranes became closely speckled with black and underwent a discoloration quantitatively more in harmony with the very marked reaction gotten with ammonia and potassium ethyl xanthate. The copper in this membrane is, therefore, also " masked "; moreover, the discovery of copper in the *Arbacia* egg membrane does not stand alone.

3. *Copper in the Chorion.*

Since the pigment during its outward passage permeates the chorion, the latter necessarily contains traces of copper. There is also some direct evidence of this, for hæmatoxylin imparts to the jelly a light bluish tint, whereas the triple-nitrite test brings out a few scattered granules stained black. Infinitesimal quantities of copper, then, can be demonstrated directly in the chorion.

4. *Copper and the Cortex.*

Inasmuch as Lillie (*loc. cit.*) has assumed the presence of a copper-avid substance in the cortex of the egg, I paid especial attention to this region both in untreated sections and in those exposed to reagents for copper.

Unquestionably the cortex differs from the remainder of the egg. Yolk, pigment, and other visible granules are absent. The zone immediately beneath the egg membrane, even at very high magnifications, seems to be optically clear. In sections not exposed to reagents for copper the cortical layer appears blue—no doubt the result chiefly of refraction. Nevertheless the color is intensified by ammonia and hæmatoxylin and does not disappear entirely when viewed by light transmitted through ground glass nor after the sections have been cleared in glycerine, xylol, or balsam.[2]

Furthermore, perhaps on account of distortions through shrinkage, the blue layer, instead of being in immediate contact with the

[2] There are, of course, other effects of refraction throughout the egg.

membrane, not infrequently lies at a considerable distance beneath
it.   All this is indicative of a region differentiated from the re-
mainder of the egg.

Is copper demonstrable in this layer?   None of the direct
methods could give an unequivocal answer, though the triple-
nitrite test did reveal a few black granules similar to the black
beads found in the membrane.   This might be considered a dem-
onstration of copper if the displacement of an occasional pigment
granule in the process of sectioning could be altogether set aside.
But there are other considerations.

The fertilization of egg fragments has been reported by numer-
ous investigators, including myself ('13[1]), yet the correctness of
these observations has been doubted because some egg fragments
are incapable of fertilization.   The difference is accounted for in
the work of Chambers,[3] who finds that fragments derived exclu-
sively from the interior of the egg can not be fertilized, whereas
those containing a fair portion of the cortex respond like the origi-
nal egg.   This evidence, if not compelling, at least indicates more
immediately than any other the physiological distinctiveness of the
cortex and the necessity of cortical materials in fertilization.

Now, certain secretions of the egg are likewise necessary.   This
was first shown by F. R. Lillie ('13[2]), and subsequently by my
sterilizations with charcoal ('21[4]).   A logical combination of our
two necessities traces the secretions to the cortex.   But are they—
or perhaps better, their forerunners—really there?   For the nor-
mal egg this has not been demonstrated, nor is the cortex, in all
probability, their only location; for egg fragments, with chorion
and membrane both removed, the inference appears less hazardous.
The concentration, then, of the secretions, or their forerunners, in
the cortex of the egg may be assumed as a not unreasonable work-
ing hypothesis.

On the basis of isolable precipitates, Miss Woodward ('18) and
I ('21[5]) have contended that these secretions contain at least two
separate substances—a lipolytic ferment and a material which ag-
glutinates spermatozoa.   The immediate problem consequently
narrows down to this: Is copper demonstrable in precipitated
lipolysin and agglutinin?

[3] See Lillie's " Problems of Fertilization," p. 264.

A. *Lipolysin—I.*

The lipolysin first tested was prepared as follows: the eggs were permitted to secrete as usual; the agglutinin was salted out with ammonium sulphate, which incidentally also precipitates much of the pigment. After filtration, the lipolysin iteslf was thrown down by means of barium, redissolved in dilute hydrochloric acid and precipitated by acetone which unless in great excess holds the remaining agglutinin in solution. The lipolysin so secured was a white powder free from pigment, though in the course of time it turned slightly purple.

After incineration a solution of the ash gave a positive reaction for copper with potassium ethyl xanthate.

Lipolysin—II.

This precipitate had exactly the same history as the foregoing except that charcoal was used in place of barium. Moreover, the powder did not change color but remained snow white and therefore may have been a somewhat purer product than lot I By the xanthate test, lot II. also contained copper.

B. *Agglutinin—I.*

The agglutinin first examined came from *Asterias* egg-secretion which had been freed from lipolysin by the barium method. The xanthate test, carried out as before, gave a perceptible reaction for copper but as the separation from the other constituents of the secretion was incomplete, this result is inconclusive.

Agglutinin—II.

A second test was made, this time with *Arbacia* agglutinin which had been merely salted out with ammonium sulphate and contained therefore considerable quantities of pigment. In this case the xanthate test was markedly positive.

Agglutinin—III.

Arbacia agglutinin whose separation from pigment and other impurities was accomplished by chloroform, charcoal, and final differential precipitation from acetone. This material was negative for copper.

In all I examined five different lots of agglutinin, and in every case preparations whose purity I supposed to be high were the ones that proved to be either completely negative for copper, doubtful, or at most very slightly positive. It appears that copper is not an essential constituent or even regular associate of pure agglutinin. These results, together with the triple-nitrite test and the considerations that suggest a cortical concentration of the secretions, justify my assumption that copper is present in the cortex of the egg immediately beneath the vitelline membrane. This copper,

however, is not associated with the egg pigment or the agglutinating material, but with the lipolysin, whose concentration in the cortex is thus one step nearer to being a demonstrated fact.

VIII. COPPER AND ENZYMES.

Lipolysin is or contains a lipolytic ferment whose presence accelerates the hydrolysis of higher and lower fats ('22[2]) and the synthesis of ethyl-butyrate ('22[3]). The association of copper with this enzyme, though contrary to expectation, can not be evaded. Does the case stand alone?

Apparently not. For example, S. Yagi ('10[2]) finds a thirty-four fold increase in the copper content of the rabbit's liver when the animal is fed 4.8 gr. copper sulphate in assimilable doses during a period of seventeen days. This agrees perfectly with the results of Titze and Wedemann ('11) on the goat and those of Rose and Bodansky ('20[1]), who find the copper content of the oyster's hepato-pancreas to be twice that of the muscle. Although as yet no one has risked the suggestion, it seems apparent that copper concentrates in tissues rich in lipases and certain other enzymes. May we risk another step and say the copper is concentrated in the enzymes? Be this as it may, it was these considerations that led me to test the following commercial preparations:

A. Pancreatin (Merck's).
> This preparation contained all the pancreatic enzymes. The ash from about .2 gr. gave a marked reaction for copper as copper xanthate.

B. Pancreatin from Pig (Squibb's).
> Result exactly as in *A*

C. Pancreatin (Parke-Davis).
> Result as in *B* and *A*.

Is the copper associated with the lipases exclusively? This can not be determined from the above tests. I therefore tried a proteolytic enzyme likely to be free from lipases, amylases, and other enzymes.

D. Pepsin (origin unknown).
> The ash from .2 gr. when freed from iron gave no positive indication for copper. However, the iron hydroxide was found to hold the copper back. I therefore washed the precipitated hydroxide with ammo

nia, whereupon the xanthate gave a very faint though unmistakably positive test.

E. Pepsin (Parke-Davis).

Also positive; faint after precipitation of copper by iron hydroxide.

These tests prove that copper occurs in preparations of pancreatic enzymes and pepsin. The association with lipolysin, therefore, is not a necessarily isolated instance, but very possibly a special illustration of a general rule. Indeed, the studies of von Euler and Svanberg ('20[2]) definitely foreshadow a natural relation between enzymes and heavy metals.

IX. LOCALIZATION OF COPPER IN EGGS EXPOSED TO COPPER SOLUTIONS.

Normal eggs, in addition to secreting copper compounds, remove copper sulphate from sea-water when this salt is present. In eggs so exposed we should expect differences, if not in distribution, then at least in the concentration of the copper which they contain. Such analyses seem doubly worth while, for they seem not only to control and correct the observations on normal material, but may ultimately throw some light on the machinery of copper fixation and metabolism in general.

Tests consequently were made on material with a total volume of .5 c.c. exposed for 20 minutes to 5 c.c. of a 1/180 *n* Cu solution in sea-water. In every other respect the eggs were treated as described in section VII. Moreover, the material was from the same lot as that used for the localization of copper in normal eggs.

1. *Copper Absorbed by the Chorion.*

From the experiments of section IV. we expect for the jelly of exposed eggs a copper content greater than that found in the normal chorion. This expectation, of course, is realized, as can be shown by means of the hæmatoxylin test with entire eggs. Superficially such eggs take on an unmistakable blue color which microscopic examination localizes distinctly in the jelly. The same effect is gotten with the triple-nitrite test, only the color is then black instead of blue.

2. *Copper Absorbed by the Egg Membrane.*

With hæmatoxylin the membrane of the exposed egg is bluer than the normal. The triple-nitrite test, however, is more convincing. In optical sections of the surface one no longer finds individual black beads strung on a thin thread, but a heavy continuous black line. Even the highest powers fail to resolve the continuity. It seems very clear, indeed, that potassium-copper-lead nitrite is more abundant than in normal membranes.

On this basis it is possible to understand one of Lillie's results. If an excess of copper in the membrane alters it so that spermatozoa are not able to penetrate as easily as under normal conditions, we should expect "a certain virtue in mass action in the presence of this inhibitor of fertilization" (*loc. cit.*, p. 129). I noted exactly the same virtue ('15) in connection with eggs which had been exposed to calcium. This particular point and its meaning have both been overlooked in subsequent discussions.

3. *Copper Absorbed by the Cytoplasm.*

Inside the exposed egg there are differences in the same sense. The number of black granules after the nitrite treatment is greatly increased. As a whole and in sections the exposed egg is distinctly darker than the normal. Copper evidently passes inward through the membrane and diffuses generally through the cytoplasm.

4. *Copper Absorbed by the Cortex.*

If, as Lillie's experiments indicate, the cortex contains "unsaturated" copper-avid material, one may expect very considerable differences in the cortical zone. As a matter of fact, the triple-nitrite test brought to light black beads similar to those seen in and immediately under the normal membrane; they were decidedly more numerous than in normal eggs and gained in distinctness by heating. Furthermore, the blue zone which in normal eggs resists resolution by all the methods employed now differentiates slightly with both potassium ethyl xanthate and potassium ferrocyanide. With the first, the blue acquires a greenish tint and becomes separated from the egg membrane by an exceedingly narrow yet distinguishable yellowish layer; with the second, a very thin brown band intervenes between the original blue and the membrane.

These changes become intelligible if we assume, in the one case, the formation of minute traces of copper xanthate; in the second, of copper ferrocyanide. Since the same reagents produce no noticeable changes in these locations in untreated material, it appears that the cortex of an egg exposed to copper contains more than the normal quantity of the metal. Moreover, the excess is localized chiefly near the surface, and since it is not the agglutinin that is copper bearing, but the lipolysin, it appears probable that the latter is also concentrated immediately under the vitelline membrane.

X. Copper Map of the Normal Egg.

From all these tests—those on the controls as well as those on material exposed to copper sulphate, I have constructed a chart which indicates the distribution of copper in the normal *Arbacia* egg. This map is a visual summary of the chief results and inferences, and in view of the preceding discussion seems to require no other comment than that given in the legend under the figure.
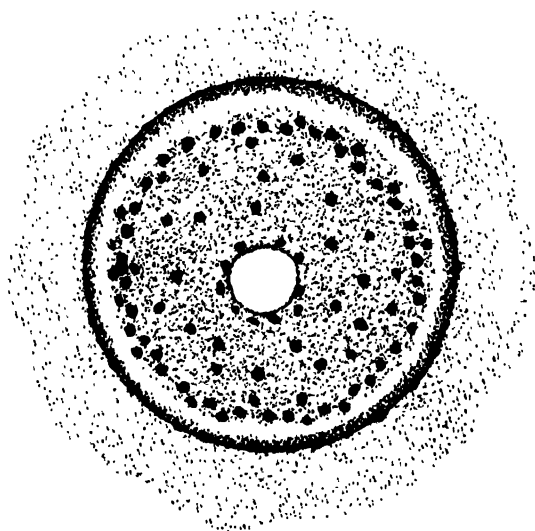

Diagram showing distribution of copper in the normal egg of *Arbacia punctulata*. The central clear area is the nucleus. Immediately about this, and extending to the cortex, the larger black spots represent pigment granules in which copper was demonstrated indirectly by analysis of secreted pigment and directly, in situ, as potassium-copper-lead nitrite. The latter, as well

as hæmatoxylin, indicates also the presence of diffuse copper in the cytoplasm. This is shown by the finer stipples.

The cortical layer is shown as differentiated into two zones: an inner, free from copper; an outer, immediately under the egg membrane with considerable concentration of copper. The evidence is again the triple-nitrite test on normal eggs, and the xanthate and ferrocyanide tests on eggs which had been exposed to solutions of copper in sea-water.

The vitelline membrane is shown as a line whose irregularly spaced black beads represent regions in which the triple nitrite was concentrated. The membranes of eggs exposed to copper solutions appear as continuous heavy black lines without beads.

The region outside the vitelline membrane represents the chorion with its diffuse and highly rarefied copper.

The diagram is intended to indicate distribution. It cannot indicate quantitative differences accurately because a variety of tests was used and the extent of discoloration depends not primarily on the quantities of copper but on the molecular size of the copper compounds formed. These are necessarily different in the several tests.

XI. ORDER OF MAGNITUDE OF THE QUANTITIES OF COPPER INVOLVED.

Quantitative data on the copper content can not be given. The methods for determining the copper are, indeed, reliable enough; but, unfortunately, the chorion alone prevents anyone from knowing how many eggs are present in 1 c.c., and until this number is definitely ascertained our initial measurements can hardly articulate closely with the results of chemical methods unusual in delicacy. For the present, then, we can arrive only at approximations.

1. *The Ovarian Egg.*

Eleven c.c. of ovary were dissolved in 11 c.c. of concentrated nitric acid and after complete destruction diluted to 100 c.c. with distilled water. Ten c.c. of this solution were evaporated to dryness and the incinerated ash dissolved. After neutralization with ammonia the volume was restored to 9 c.c. To this was added 1 c.c. of a solution of potassium ethyl xanthate whose concentration was 1 gr. per 1,000 c.c. of distilled water. This same amount was added to 9 c.c. of a copper sulphate solution containing 10 μ gr. of copper per c.c.

Colorimetric comparisons were then made, and the concentration, C_2, of the unknown calculated from the concentration, C_1, of the standard and the depth of the unknown, D_2, necessary to match

a given depth, D_1, of the standard.   Accordingly,

$$C_2 = \frac{C_1 \times D_1}{D_2}.$$

Test	$\dfrac{C_1 \times D_1}{D_2}$	Micrograms Cu. per c.c. Eggs.
I........	$\dfrac{10 \times .50}{2.21}$	20.54
I........	$\dfrac{10 \times .30}{1.38}$	19.76
II.......	$\dfrac{10 \times 2}{1.40}$	14.29
II.......	$\dfrac{10 \times 2}{1.31}$	15.27
III.......	$\dfrac{10 \times 1.5}{1.12}$	13.39

Average $=$ 16.65 μgr.

2. *The Shed Egg.*

Ten c.c. of the material referred to as *A* in section VI. were treated and tested in exactly the same way.

Test	$\dfrac{C_1 \times D_1}{D_2}$	Micrograms Cu. per c.c. Eggs.
I.........	$\dfrac{10 \times 1}{1.55}$	161.25
I.........	$\dfrac{10 \times 1.5}{2.2}$	170.50
II.........	$\dfrac{10 \times 1}{1.40}$	178.50
II.........	$\dfrac{10 \times 1}{1.33}$	188.00

Average $=$ 174.56 μgr.

3. *The Fertilized Egg.*

Forty c.c. from a solution of 1.2 c.c. of fertilized eggs gave the following results:

Test	$\dfrac{C_1 \times D_1}{D_2}$	Micrograms Cu. per c.c. Eggs.
I..........	$\dfrac{10 \times .5}{2.00}$	20.83
I..........	$\dfrac{10 \times .5}{1.96}$	21.25
I..........	$\dfrac{10 \times .5}{2.03}$	20.50

Average $=$ 20.86 μgr.

The copper content of the fertilized ovum apparently has the general order of magnitude characteristic of the unshed ovarian egg. That of the shed egg is, roughly, from eight to ten times greater.

XII. DISCUSSION.

1. *The Copper Problem.*

Unless bound up in respiratory or other pigments, copper is considered essentially a poison. Its use in germicides and fungicidal solutions, the harmful influences on higher and lower plants, power to block organic catalysis, the medicinal properties of colloidal suspensions and copper salves—all support the prevalent view. Yet there are no poisons in nature; there are only poisonous effects. These may be exercised by the commonest articles of diet at certain concentrations and by copper, it happens, at very low ones.

Even a cursory examination of the literature suggests further misgivings. Copper has been found repeatedly in three of the five main divisions of the plant kingdom and among animals, with equal frequency, in nine phyla, ranging from protozoa—if *Volvox* ('21[6]) is an animal—to man. However, only Maquenne and Demoussy ('20[3]), who worked on plants, and Rose and Bodansky ('20[1]), whose studies cover a wide range of marine animals, are actually bold enough to suggest that copper in general may be more than an adventitious element which living things somehow tolerate.

Specific processes in which it normally plays a rôle are still unknown; moreover, they are quite likely to remain so unless we free ourselves for investigation by an admission of ignorance.

As a matter of fact, copper has many of the qualifications of a

biological element. It is widely diffused in the environment and has been so since earliest geological time. Though listed among the less active metals, it is capable of entering into a large variety of combinations, including numerous organic unions. It is difficult to see how a living thing could avoid copper except by some definite mechanism of exclusion.

The absence of this is significant, but does not establish physiological importance. Very possibly living things are mere sieves that hold the copper back; very possibly, too, its marked concentration in such tissues as the liver is a liability, by accident dependent on other attributes and under ordinary circumstances negligible. Still one would like to be certain, and in our present state of knowledge this is impossible. Yet a negative answer, excluding copper from the realm of physiological processes, even now appears unlikely. It is impossible to conceive the synthesis of respiratory pigments, turacin, or any other product without thinking of a long linkage of reactions inevitably affected, directly or otherwise, by their final end result.

2. Copper and Enzymes.

Von Euler and Svanberg (*loc. cit.*) have shown that the addition of saccharase results in a marked reduction of the free silver ions in dilute solutions of silver nitrate. Since the silver did not become colloidal under the influence of reductions, possibly due to certain constituents of the enzyme preparation, these writers suggest a binding of the silver ions to certain constituents of the saccharase solution.

Since such unions render the enzyme inactive, why not assume that the constituent of the solution with which the metallic ions combine is the enzyme itself, an organic co-enzyme, or both? Such an assumption seems all the more reasonable because prolonged dialysis, I find, does not remove all the copper. Thus if copper incapacitates at all, catalysis would be excluded in any case.[4]

[4] It is not possible that certain differences of proportionality between the effects of silver and mercury salts could be explained on this basis? Perhaps too the recovery known as " The Danysz Phenomenon " results from a redistribution of metallic ions between enzyme and co-enzyme. Even if co-enzyme and enzyme together make up, and are identical with, the entity, enzyme, the allocation of Cu to different positions in this system might have results essentially those suggested both in sense and in degree.

This assumption involves on the part of enzymes or organic co-
enzymes a capacity for combining with heavy metals. Since this
power is not only very great, to judge by the dilutions that in-
capacitate, but likewise inseparable from normal ferments, we
should not be astonished if enzymes from copper-bearing organ-
isms or tissues give positive results when tested for copper.

3. The Oligodynamic Effects of Copper.

The poisonous effects of extraordinarily high dilutions of metal-
lic copper were first noticed by Nägeli ('93), who found one part
in seventy-seven millions rapidly fatal to *Spirogyra*. Because of
the extremely small quantities involved Nägeli spoke of "oligo-
dynamic action."

As these results have been repeatedly verified on other forms, it
may be convenient to retain Nägeli's term. However, "oligo-
dynamic" need imply nothing more mystical than a chemical or
perhaps physical relation between extremely small quantities of
material. As yet no acceptable explanation has been offered.[5]

But there have been suggestions. Nägeli was able to destroy
the toxicity of oligodynamic solutions by means of paper, wool,
paraffin, gums, and gelatin. The experience is quite comparable
to the protective action of egg-water, gum arabic, and gelatin de-
scribed by Lillie ('21[2]). Inasmuch as the copper in the first case
at least is probably present as electropositive colloidal hydroxide
or carbonate, Bayliss ('15[2]) imagines adsorption on the electro-
negative surfaces of Nägeli's detoxicants as the basis of their anti-
toxic properties. Conversely, the toxic effects become explicable
as the outcome of adsorption by electronegative surfaces or parti-
cles critically involved in or on the affected tissues.

At this point Bayliss leaves the problem. We still do not know
why such minute quantities are effective. Do they cause precipi-
tation? If so, how much precipitation can they conceivably bring
about? And, finally, what is precipitated? The neutralization of
electrical charges in all likelihood is unavoidably associated with

[5] Quite recently, Saxl, as quoted by von Euler and Svanberg, assumed "dass
die Metalle eine eigenartige Keimtötende Kraft besitzen, welche nicht mit der
Löslichkeit der Metalle zusammen hängen soll." Saxl refers to a physical
energy "die sich zunächst auf der Oberfläche der Metalle abspielt, jedoch
auch in andere Medien übergehen kann" ('20[2] p. 378).

the ultimate mechanism of oligodynamic activity.   For the present, however, our concern must be with facts a trifle more immediate.   From these, it seems to me, we can derive a second suggestion.

The catalytic power of colloidal platinum is destroyed by traces of hydrocyanic acid and can be restored by simple aëration. Moore ('21[7]) compares this experience of Bredig and his pupils with the effects of hydrocyanic acid on the peroxidases of the enzymic oxidation system.   " If these peroxidases are responsible in tissue cells for the uptake of oxygen by the protoplasm, it may well be that the poisonous action of hydrocyanic acid in such minute doses is due to interference with the action of the peroxidases " (loc. cit., p. 231).

Disregarding the specific case which Moore had in mind, there is implicit here a definite relationship between enzymes and oligodynamic action.   This relation, however, is also implicit in the copper inhibitions of Lillie.   Again the union of saccharase with silver or copper implied by von Euler and Svanberg, together with the natural occurrence of copper in preparations of lipolysin, pancreatic enzymes, and pepsin, now reported, I believe, for the first time, are just what one might expect if the oligodynamic properties of heavy metals are traceable to the activation or inactivation of enzymes.   These effects must depend on the capacity of enzymes to bind the metal.   Until stoichiometrical determinations have been made nothing further can be said about such unions. But no matter what their nature may be, their occurrence should result in the presence of heavy metals in enzyme preparations whenever such presence is a physical possibility.

But the presence of copper raises an apparent difficulty.   One might arrive at the paradox that enzymes, by the very nature of the case, must be normally incapacitated.   This absurdity is easily dispelled.   There is no reason for considering the normal enzyme as saturated with copper.   For that matter, the enzyme proper may not be involved at all, for the observed effects can all be explained equally well if the copper is held by organic co-enzymes where these are present.   But in this case also we are not compelled to consider the co-enzymes as saturated.

On this basis it is possible to explain the most puzzling feature of oligodynamic action. It is not that copper, silver, gold, or any other metals have this or that effect, but that infinitesimal amounts give results apparently out of all proportion to the quantities involved.

In the case of copper, for example, I imagine the oligodynamic effect as due to the inactivation of that fraction of the enzyme or co-enzyme which was not inactivated by the copper present in the first place. Where the oligodynamic effect is produced by silver or some other metal, the total of inactivated enzyme or co-enzyme would be composed of two fractions—the one inactivated by copper, the other by silver.

This view of the case has stoichiometrical implications which further work may or may not justify. But however this may turn out, the presence and effect of copper in preparations of normal enzymes calls urgently for further study.

Quite apart from the biochemical questions that suggest themselves at once, the discovery carries with it problems of wide biological significance. Is the liability to inactivation compensated by the production of larger quantities of the enzymes? Or does compensation come about by variety and differential susceptibilities? Why the tremendous number of enzymes when, aside from a few cases of molecular rearrangement, the only radical processes we have to deal with are the reversible oxidations and hydrolyses?

4. Copper and Fertilization.

Probably most of the copper present in the *Arbacia* egg is incorporated in the pigment whose elimination discolors the sea-water. As I have shown ('14), the rate of pigment secretion is increased 50 per cent. while the eggs are undergoing fertilization. This explains why the copper content of fertilized eggs is so much lower than that of unfertilized. My values for the copper content indicate in fertilized eggs an order of magnitude quite different from that in ripe eggs and essentially the same as that of the immature ovarian ovum. Tentatively, therefore, one may hazard that, among other things, fertilization restores the copper content to an order of magnitude characteristic of the unripe egg.

In the absence of more accurate data it is premature to discuss

this fact at length.   Nevertheless we may possibly find along these lines some help in clearing up the uncertainties that now beset us. Very possibly the concentration of copper normally has something to do with checking the growth of the egg, whereas the heightened rate of secretion during fertilization restores conditions essential for further growth and development.   No doubt the linkage can be pictured in several ways, but one way is this: the elimination of pigment from the egg might result in the production of fresh pigment, or some other product, which, if copper-avid, might also draw upon the copper of the enzymes and thus assist in the process of activation.

All this, however, is merely in the realm of possibilities.   At the rate at which discoveries are being made in this field hardly anyone would wish to formulate a theory of fertilization.   The process is far more complicated than it seemed ten years ago, and if one thing is ·more certain than another, it is that the major classes of evidence have not yet been handed in.

XIII.   SUMMARY.

1.   Nearly 37 per cent. of the copper sulphate added to sea-water is precipitated at once when the concentration is $Cu = n/460$.

2.   Two tenths c.c. normal *Arbacia* eggs in 75 minutes reduce the concentration of 14.8 c.c. of a Cu solution from $n/1,460$ to $n/1,790.9$.

3.   In this reduction the egg jelly or chorion is heavily involved.

4.   It was impossible to determine the quantities of copper absorbed by normal eggs and eggs without jelly because of an apparent secretion of copper by the eggs themselves.

5.   Therefore, the demonstration of copper in *Arbacia* eggs was undertaken.   The copper was identified as cupric hydroxide, cupric cyanide, cupric ferrocyanide, copper xanthate, crystalline cupric sulphate, as metallic copper on tin-foil and aluminum, and finally by electrolytic precipitation under conditions under which copper and only copper could be deposited.

6.   The copper was localized in the egg directly by means of hæmatoxylin and the triple-nitrite of potassium-copper and lead. Indirectly it was localized by the analysis of egg secretions.

7.   The copper occurs chiefly in the egg pigment; the vitelline

membrane and the chorion also contain copper.   It is not a regular constituent of agglutinin precipitates, but was found constantly in precipitates of lipolysin.

8. This association with lipolysin makes possible the localization of both copper and ferment in the cortex of the egg.

9. The association of copper with lipolysin is not an isolated case.   Copper was found also in preparations of pancreatic enzymes and pepsin.

10. In eggs exposed to a $n/180$ Cu solution for twenty minutes the copper is widely diffused through the cytoplasm and concentrated in the chorion, the vitelline membrane, and the cortex.

11. Approximately the amounts of copper normally present in 1 c.c. of *Arbacia* eggs are as follows:

> Unripe ovarian eggs $=$ 17 μ gr.
> Ripe shed eggs $=$ 175 μ gr.
> Fertilized eggs $=$ 21 μ gr.

12. From the preceding and other considerations it is suggested that copper, in general, may be more than an adventitious element, physiological only in pigments, and merely tolerated in all other connections.

13. The association of copper with enzymes is explained as the outcome of some sort of union, very likely chemical, between enzymes or co-enzymes, or both, and the metal.   It is also suggested that possibly the differences in the proportionality between silver and mercury effects, as well as the Danysz recovery after "poisoning," may be due to the distribution and subsequent redistribution of the metallic ions between enzymes and co-enzymes.

14. The oligodynamic action of copper is explained as due to the inactivation of that fraction of the enzyme or co-enzyme which was not normally inactivated by the copper present in the first place.   If inactivation is produced by silver, it is suggested that the total inactive enzyme or co-enzyme would be composed of two fractions—the one inactive because of the normal copper content, the other because of the silver added.

15. It is suggested that the concentration of copper in the ovum at maturity may have something to do with limiting the growth of the egg; that the elimination of copper-bearing pigment during

fertilization may indirectly restore or produce conditions essential
for further growth and development.

XIV. LITERATURE.

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BIOLOGICAL BULLETIN

THE TEMPERATURE COEFFICIENT OF A HETERO-
ZYGOTE WITH AN EXPRESSION FOR THE VALUE
OF A GERMINAL DIFFERENCE IN TERMS OF AN
ENVIRONMENTAL ONE.[1]

CHARLES ZELENY.

It has been shown by Seyster and Krafka that the size of the
eye and the number of its ommatidia in "bar-eye" *Drosophila*
varies with the temperature at which the larvæ are developed. An
increase of one degree Centigrade produces on the average a de-
crease of about 10 per cent. in ommatidial number. In ultra-bar,
an allelomorph of bar and full, the change is about 8 per cent. per
degree. On the other hand, full eye has a much lower temperature
coefficient. Counts being made at present by Miss Karrer show
a change of only $2\frac{1}{2}$ per cent. per degree.

Since the effect upon bar and ultra-bar is so much different from
that upon full, it becomes a matter of interest to determine the
reaction of the heterozygotes. Are they intermediate in this re-
spect as well as in ommatidial number? The results may be ex-
pected to throw some light upon the manner of reaction of the
genes and on the nature of dominance.

The present report deals with the ultra-bar heterozygotes.

The effect of temperature upon ultra-bar homozygotes has been
determined by Krafka (1920, p. 416). His values are copied in
the next to the lowest line of Table I. From these values the
average effect of one degree of change in temperature may be
determined as follows: The average ommatidial number is 51.5 at
15° and 15.8 at 30°. The difference between the logarithms of
51.5 and 15.8 divided by fifteen and reduced to its arithmetical

[1] Contribution from the Zoölogical Laboratory of the University of Illinois.
No. 210.

value is 1.080. In other words, one degree decrease in temperature between 30° and 15° causes an average increase of 8 per cent. in ommatidial number. Between 27° and 15° the average increase is 7.6 per cent.
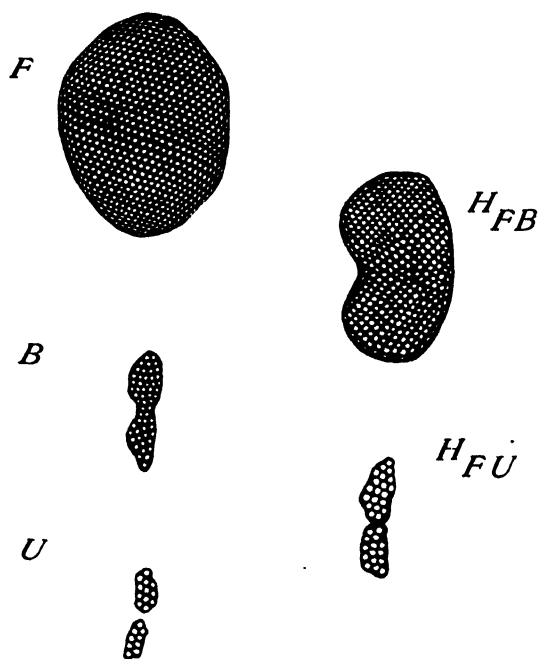
Fig. 1. *F, full eye. B,* bar. *U,* ultra-bar. H_{FB}, heterozygote of full and bar. H_{FU}, heterozygote of full and ultra-bar.

In some work not yet published, but which she has allowed me to use for the present purpose, Miss Karrer finds that full eye has an average increase of only about 2½ per cent. per degree between 29° and 15°.

TABLE I.

	Number of Ommatidia.			
	15.	**20.**	**27.**	**30.**
Two full eye factors. Homozygous full eye..........................	1,077.5	947.0	810.6	754.9
One full eye and one ultra-bar factor. Heterozygote....................	112.1	72.1	40.5	37.1
Two ultra-bar factors. Homozygous ultra-bar........................	51.5	32.6	21.3	15.8
Difference between heterozygote and homozygous ultra-bar............	60.6	39.5	19.2	21.3

Heterozygotes Exhibit a Complete Change in Reaction from the Full to the Ultra-bar Type.—In the case of heterozygous females, containing one full and one ultra-bar factor, the mean ommatidial values as determined by the present experiments are given in the second line of Table I. There are 37.1 ommatidia at 30°, 40.5 at 27°, and 112.1 at 15°. The average increase per degree determined by the method previously described is 7.7 per cent. between 30° and 15° and 8.9 per cent. between 27° and 15°. These values are strikingly different from 2.5 per cent., the value for full eye, and strikingly similar to 8.0 and 7.6, the values for homozygous ultra-bar.

The temperature coefficient of the heterozygotes must, therefore, be considered as essentially like that of the ultra-bar parent and wholly different from that of the full parent. While a single ultra-bar factor is not sufficient to bring about the complete effect in reducing ommatidial number as produced by two ultra-bar factors, it is sufficient to produce the complete change to a physiological system of the ultra-bar type.

The change in ommatidial number with change in temperature can be explained most satisfactorily by assuming a differential effect of temperature upon the physiological processes involved in ommatidial production as opposed to other physiological processes. In view of the fact that temperature is effective only during a few hours of larval life, it may be considered that the initial steps in the formation of ommatidia are confined to a definite embryological period. The length of this period is determined by the general physiological processes of the larva, while the rate of formation of ommatidia during the period is a function of special processes which have a different coefficient. It is evident that under these circumstances two different temperatures must give two different ommatidial numbers. The difference in the temperature coefficients is slight in full eye and the mutation to bar or ultra-bar involves a marked increase of this difference. Further analysis awaits a more accurate knowledge of the nature of the embryological processes involved. Whatever the character of these processes, however, it is clear that the reaction system produced by the introduction of a single ultra-bar factor is of the ultra-bar type, even though the reduction in ommatidial count at any specific tem-

perature is not as great as that produced by two ultra-bar factors.

The analysis makes it evident that there are two distinct processes involved in the mutation from full to ultra-bar. One of these consists in an essential change in the type of reaction as shown by the change in the temperature coefficient. The other process involves a change in the general level of the rate of the reaction without affecting its specific character. The first is fully accomplished by a single ultra-bar factor. The second is influenced quantitatively by the number of ultra-bar factors.

The Effect of Temperature upon Dominance.—Since the heterozygote has the same temperature coefficient as homozygous ultra-bar, and one that is much greater than that of homozygous full eye, it becomes a matter of interest to consider the effect of temperature upon dominance. Elsewhere (1920, p. 308) I have discussed a method of determination of the coefficient of dominance by the use of a factorial scale in which the effect of a degree of temperature is taken as the measure of an unit factor. It is obvious that on this basis there can be no change in dominance with temperature, because the ommatidial value of the unit varies with change in effect of temperature.

If, however, it is desired to get an expression for dominance which is based directly upon the somatic expression—*i.e.*, upon the ommatidial number—such a value changes with the temperature. Suppose that complete or 100 per cent. dominance of full is a condition in which the heterozygote has the same ommatidial number as full eye and complete recessiveness or zero per cent. dominance of full a condition in which the heterozygote has the same ommatidial number as ultra-bar. Likewise suppose that the ommatidial count is the scale of values. Then the coefficient of dominance of full as expressed on a percentage basis is

$$\text{C.D. } F = \frac{H - B'u}{F - B'u} \times 100,$$

in which H is the ommatidial count of the heterozygote, $B'u$ that of ultra-bar, and F that of full. Correspondingly

$$\text{C.D. } B'u = \frac{F - H}{F - B'u} \times 100.$$

In this way the coefficient of dominance of full eye is readily deter-
mined as 5.9 per cent. at 15°, 4.3 per cent. at 20°, 2.4 per cent. at
27°, and 2.9 per cent. at 30°.   The general decrease with increase
in temperature is evident.   The reversal between 27 and 30 may
probably be explained by the disturbance resulting from an ap-
proach to the maximum temperature.

*The Value of a Germinal Factor in Terms of an Environmental
One.*—Perhaps the most interesting point in connection with the
present data is the demonstration that they furnish of the fact that
the gene, ultra-bar, has the same type of reaction as a temperature
difference.   It is possible to state the effectiveness of particular
germinal factors in terms of the corresponding effects of tempera-
ture.   Such an attempt has been made in previous studies of the
bar races and the temperature coefficient has given the basis for
the evaluation.   Since these previous studies had shown that a
change of one degree in temperature produces a change of approxi-
mately 10 per cent. in ommatidial number, a " unit " factor was, for
convenience, taken as one that produces the same change.   The
units in the factorial scale, whether environmental or germinal, are
thus expressed on the same basis.

First of all, it will be well to take up the demonstration of the
fact that the particular germinal difference represented by the
addition of a second ultra-bar factor in place of the full factor of
the heterozygote does not correspond to a constant somatic expres-
sion.   The difference between heterozygous and homozygous ultra-
bar is not represented by a constant difference in ommatidial num-
ber.   Thus at 15 degrees the difference is 60.6 ommatidia, at 20
degrees 39.5, at 27 degrees 19.2, and at 30 degrees 21.3.   These
data are given in Table I. and in graphic form in Fig. 2, where the
lengths of the heavy vertical lines are proportional to the omma-
tidial differences at the various temperatures.   The marked change
with temperature is obvious, though the germinal difference re-
mains constant.   The ommatidial difference can not, therefore,
serve directly as a measure of germinal difference.

If, however, the unit of measurement is the effect produced by
a degree of change in temperature, 8 per cent. in this case, and the
effect produced by the substitution of the second ultra-bar factor
for the full factor of the heterozygote is measured in terms of this

unit, the result obtained is shown in Table II. and graphically in
Fig. 3. In the latter the scale at the left represents the logarithms
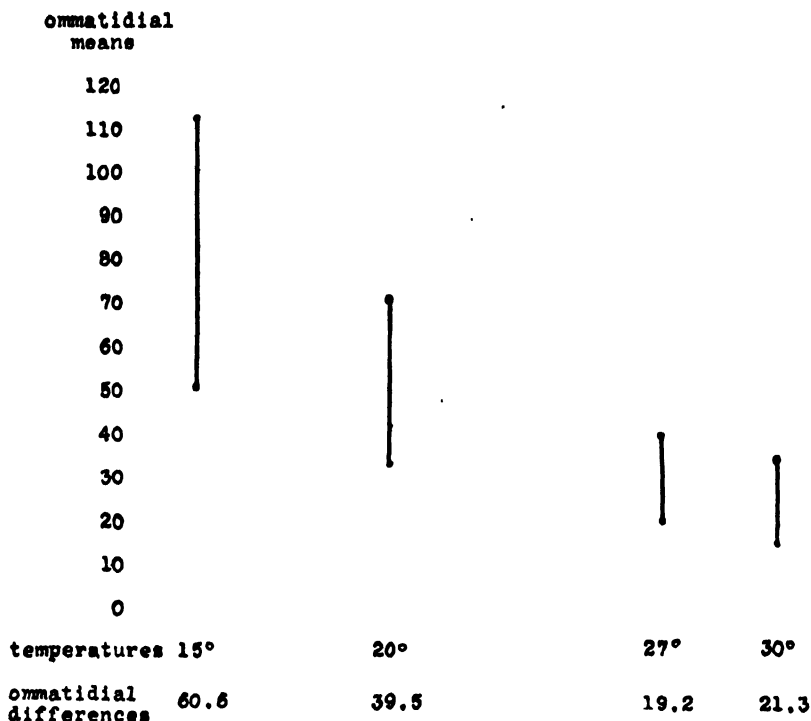
FIG. 2. The length of each heavy vertical line represents the difference in
ommatidial number between homozygous and heterozygous ultra-bar at the
respective temperature. The numerical values are given at the bottom of
the figure. The heterozygotes have one full and one ultra-bar factor. The
scale at the left represents ommatidial numbers. The marked change in the
ommatidial difference with change in temperature is to be noted.

TABLE II.

FACTORIAL VALUES.

	Eight Per Cent Units.			
	15°	20°	27°	30°
One full eye and one ultra-bar factor. Heterozygote....................	+25.3	+19.6	+12.3	+11.0
Two ultra-bar factors. Homozygous ultra-bar........................	+15.3	+ 9.4	+ 3.9	0.0
Difference in eight per cent. units or temperature units................	10.3	10.2	8.4	11.0

of the ommatidial counts, arranged so that each unit has the same logarithmic value, corresponding to a change of 8 per cent. in ommatidial number. The location of the zero point is of course purely arbitrary.

logs of ommatidial means	scale of degrees				
2.034	+25				
1.868	+20				
1.700	+15				
1.533	+10				
1.366	+ 5				
1.199	0				
temperatures		15°	20°	27°	30°
log. differences		0.338	0.345	0.279	0.371
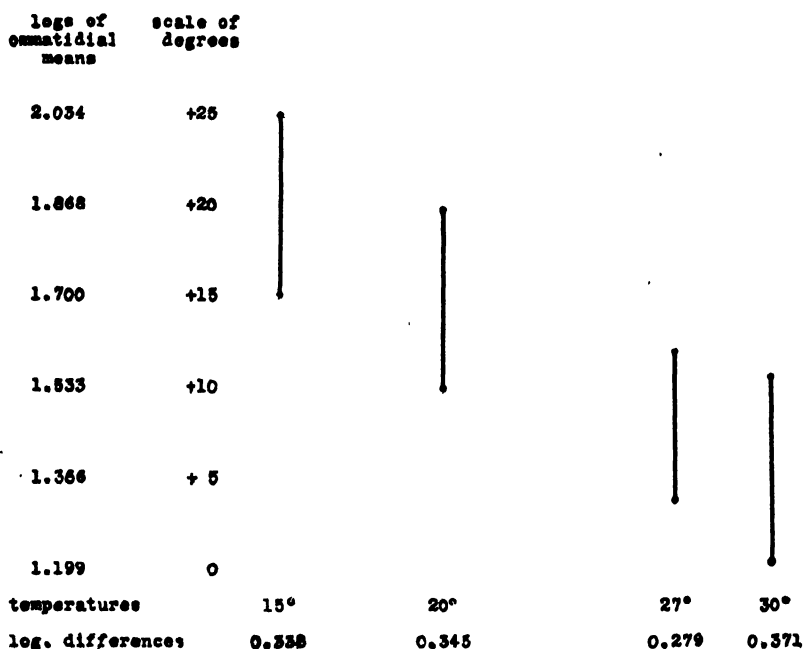
FIG. 3. The length of each heavy vertical line represents the difference in terms of logarithms of ommatidial number between homozygous and hetero-zygous ultra-bar at the respective temperatures. The numerical values are given at the bottom of the figure. The heterozygotes have one full and one ultra-bar factor. The logarithmic scale is represented at the left. In the next column is the scale of corresponding degrees Centigrade which would produce the same effect, starting with an arbitrary zero at the lowest value of the present observations. It is to be noted that the difference between homozygote and heterozygote on this scale is fairly constant.

The heavy vertical. lines again give the effect produced by the substitution of a second ultra-bar factor for the full factor of the heterozygote at the different temperatures. The second ultra-bar factor at 15° C. depresses the value by an amount equal to that produced by 10.3 degrees of temperature, at 20° C. by 10.2 degrees, at 27° C. by 8.4 degrees, and at 30° C. by 11.0 degrees. The average depression is the same as that produced by ten degrees of increase in temperature, and considering the character of

the determinations the values are remarkably constant. The conclusion may be safely made, therefore, that a proper measure has been found for the expression of the germinal value in question. A homozygous ultra-bar at 15° has the same ommatidial number as a heterozygote at 25° and a homozygote at 20° the same number as a heterozygote at 30°.

On the logarithmic scale of ommatidial number it is, therefore, possible to express the relation between the germinal factor and the environmental factor as a constant. The present data, therefore, strengthen the validity of the use of this scale as a measure of the germinal factors as well as the environmental ones.

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STUDIES ON THE PHYSIOLOGY OF RECONSTITU-
TION IN *PLANARIA LATA*, WITH A DESCRIP-
TION OF THE SPECIES.

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The earlier work on the reconstitution of isolated pieces of planarians into new individuals was very largely concerned with the description of the visible changes, such as the outgrowth of new tissue, its differentiation, the reorganization and redifferentiation of the old parts, the changes in shape, the minimum size of pieces capable of reconstitution, and the relations between the polarity of the new individual and that of the animal from which the piece was taken. Most of the experiments were performed with small numbers of pieces, often from different regions of the body and without any attempt at a physiological standardization of the experimental material. During the past twenty years *Planaria dorotocephala* has been extensively used in this laboratory as material for experimental investigation, and in the course of this work various methods have been developed which have made possible some degree of physiological analysis of the process of reconstitution in this species. In this work physiological standardization of material, mass experiments, and control of environmental conditions have played an important part.

The desirability of including other species within the program of investigation has become increasingly evident with the progress of the work on *P. dorotocephala,* and at the suggestion of Dr. C. M. Child the analysis of reconstitution in a closely related species was begun. The present paper comprises a part of the results of this investigation.

In this connection I take the opportunity to acknowledge my deep indebtedness to Professor Child for his advice, friendly criticism, and revision of the manuscript. I also wish to express my thanks to Dr. L. H. Hyman for the data on oxygen consumption presented in this paper and for many helpful suggestions.

MATERIAL: DESCRIPTION OF SPECIES.

In many rivers and lakes about Chicago a planarian, commonly identified in the past as *P. maculata,* occurs in large numbers. Even a cursory examination makes it evident that this form differs in various respects from *P. maculata* of the Atlantic slope. Attention has already been called to these differences by Hyman ('20). A comparison of living individuals of this form and *P. maculata* from the region of Woods Hole, Mass., shows the following differences: The pigment pattern of the mid-western form (Fig. 1) is distinctly coarser and more irregular; the individual pigment spots and the unpigmented areas are more clearly visible to the naked eye than in *P. maculata* (Fig. 2). In a mixed stock the two
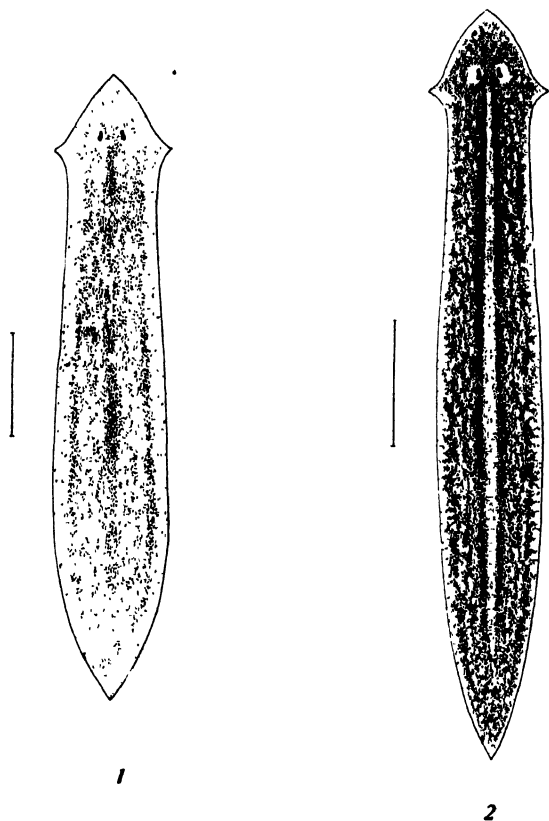
FIG. 1. *Planaria lata* n. sp.

FIG. 2. *Planaria maculata.*

forms are at once distinguishable by these differences in pigmentation. Moreover, the general color effect to the naked eye in the mid-western form is a light grayish brown, mottled or dappled, while in *P. maculata* the brown tint is much deeper and more uniform. In *P. maculata* a light median longitudinal stripe is almost invariably present (Fig. 2), while the mid-western form usually shows an obscure dark median stripe (Fig. 1).

As regards shape of body, the mid-western form is distinctly broader in proportion to length than *P. maculata* (cf. Figs. 1 and 2). This difference is evident during locomotion as well as at rest. The cephalic lobes are apparently slightly less developed and the digestive tract appears more highly branched in the mid-western form than in *P. maculata*.

As regards motor behavior, also marked differences appear. The mid-western form is distinctly more sluggish, reacts more slowly, and progresses less rapidly than *P. maculata*. Similar differences in reaction to food exist. *P. maculata* can be collected by placing pieces of meat in the water in localities where the species occurs. As the extractives diffuse, the animals will come to the meat from a distance of several inches in standing water and from greater distances in flowing water. The mid-western form can not be collected in any considerable numbers in this way because only those animals immediately about the meat react. In feeding stocks in the laboratory it has been found necessary to grind the meat and spread it over the bottom of the container, instead of placing pieces at intervals of two or three inches, as has been the practice with *P. dorotocephala* and *P. maculata*.

All these differences persist unchanged in stocks of the two forms kept in the same water and under the same conditions in Chicago. This is true, not only as regards the original individuals, but also as regards young animals resulting from fission or reconstitution of pieces and animals hatched from eggs. Reconstitution experiments with the two forms also show certain characteristic differences in head frequencies.

These differences are unquestionably sufficient to make it evident that the mid-western form is not *P. maculata*. However, since the morphological characteristics of the organ complex of the genital atrium, and particularly the copulatory organ, are com-

monly regarded as the most trustworthy criterion of specific differences, sections of this region of sexually mature animals have been made.
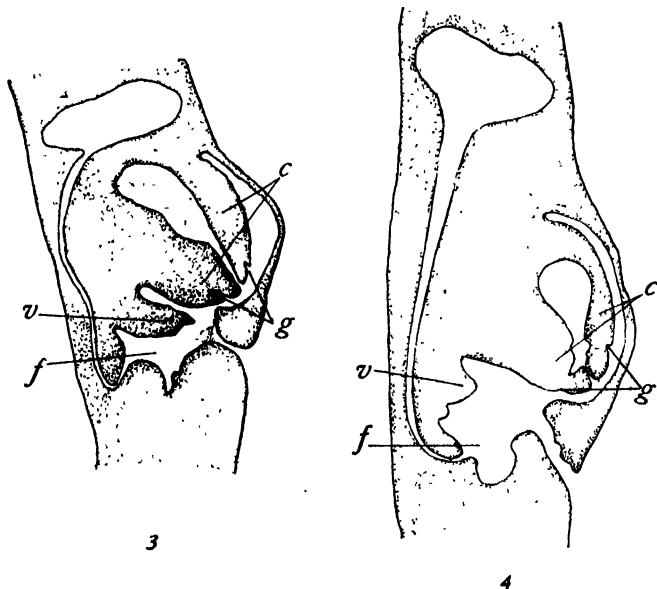
FIGS. 3, 4. Atrial genital complex of the two species of *Planaria*: Fig. 3, *P. lata*; Fig. 4, *P. maculata* combined from two adjoining sections; *c*, copulatory organ; *f*, female region of atrium; *g*, circular groove or furrow on copulatory organ; *m*, male region of atrium; *v*, valve or fold between the two parts of atrium.

Fig. 3 is a median sagittal section through this region of the mid-western form and Fig. 4 a similar section from *P. maculata*, both from sexually mature animals. The circular groove (*g*) about the apical region of the copulatory organ (*c*) is deeper in *P. lata* and the outline of the fold or valve (*v*) between the common portion of the atrium and the female duct (*f*) is very different in the two species. Other differences in shape of the parts and cavities are evident, but may be due in part to differences in muscular contraction. The differences in the atrial complex confirm the conclusion that the two forms are different species, and since the mid-western form does not agree with descriptions of other species already given, it is evidently an unnamed species and is named and described as follows:

Planaria lata n. sp.—Length of full-grown, sexually mature in-

dividuals averaging 12–14 mm., occasionally 16 mm. Body relatively broad, in full-grown animals width from tip to tip of cephalic lobes and from margin to margin at mouth about one sixth of length. Pigment pattern coarse, irregular, often with obscure dark median stripe, the general effect being mottled or dappled light grayish brown. Cephalic lobes short. Animal sluggish and markedly less sensitive to external factors than either *P. dorotocephala* or *P. maculata.* Structure of organ complex of genital atrium as in Fig. 3.

The present paper is primarily concerned with this species, but attention is called to the physiological differences between this species and *P. dorotocepha* which have been brought to light by the experiments.

The Occurrence of Fission and Sexual Maturity.

Like *P. dorotocephala, P. lata* shows no visible morphological indication of the presence of a posterior zoöid, but, as will appear below, the presence of such a zoöid can be demonstrated by physiological methods. Fission in *P. maculata* has been described by Curtis ('02) and the act of fission has been observed in *P. dorotocephala* by Child ('10, '11c). In the latter species it consists in an independent motor reaction of the posterior zoöid while the animal is moving forward. The posterior zoöid attaches itself, the anterior zoöid continues to advance, and the body in front of the attached portion is finally ruptured. Fission is much more likely to occur after slight stimulation than after violent disturbance, for in the latter case the posterior zoöid is controlled by the anterior and does not react independently. The act of fission has not been observed in *P. lata,* but undoubtedly it occurs in the same manner. In the laboratory fission is often induced by changing the water, but does not occur at once, while the animals are very active, but only later as their activity decreases.

As regards the level of the body at which fission occurs, *P. lata* differs markedly from *P. dorotocephala.* In the latter species fission normally occurs at a level 1–3 mm. posterior to the mouth (Fig. 5), and in cases of delayed fission in the laboratory the posterior fission piece may be longer than the anterior. In *P. lata,* however, fission takes place much nearer the posterior end (Fig. 6).

and in animals 12–13 mm. in length the posterior fission piece is only 2–3 mm. in length, and in shorter animals it is not only abso-
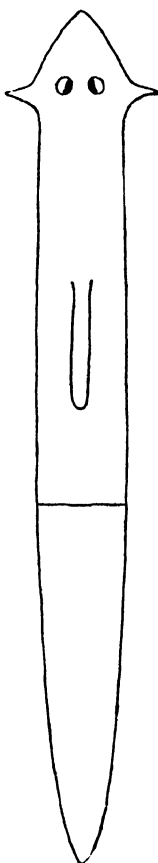
FIG. 5. *P. dorotocephala,* showing level at which fission usually occurs.

FIG. 6. *P. lata,* showing level at which fission usually occurs.

lutely, but relatively, shorter.   In *P. dorotocephala* the posterior fission piece often divides again after four or five days, when it begins to move about more or less normally.   Apparently at this stage the developing head is unable to control the whole length of the posterior fission piece and fission takes place at one of the more posterior zoöid boundaries.   Such second fission has never been

observed in *P. lata,* but the susceptibility (p. 32) and the head frequency (p. 37) of the region just anterior to the level of fission suggest that some slight degree of physiological isolation and the earliest stages of another zoöid exist there in large animals. If such a zoöid is present, it develops to a stage at which fission is possible only after fission has occurred posterior to it and it has become the posterior end of the body.

The maximum length attained by *P. lata* under ordinary conditions in nature and in the laboratory is twelve to fourteen millimeters, and at this size the animals become sexually mature, even though fission continues to occur. The level of fission is so far posterior to the genital pore that the development of the ducts and pore is usually not affected to any appreciable degree by fission. In this respect also this form differs from *P. dorotocephala,* in which the level of fission is so near the level of the genital pore that the occurrence of fission in an animal approaching sexual maturity usually brings about disappearance of the pore and at least the posterior portions of the ducts. In the localities about Chicago *P. lata* becomes sexually mature and deposits eggs from June to September. In the laboratory maturity and egg laying may occur at any time of year. In *P. dorotocephala* sexual maturity may occur in the laboratory when the animals are well fed and fission is prevented by keeping them on slimy or vaselined surfaces, but it has not been observed under natural conditions in this region, being apparently prevented by fission and perhaps also by periodic starvation (Child, '11c). Curtis also found that in some localities *P. maculata* does not become sexually mature, but did not discover the determining conditions. It may be suggested that an environment which inhibits slightly the physiological activity of the animals and so decreases the range of dominance may result in the occurrence of fission at a more anterior level, and this may interfere with the development of the genital ducts and pore. Experiments to test this suggestion have not yet been performed.

METHODS.

The animals are found both on stones at the bottom and on *Elodea* and other water plants at various levels. On the stones all sizes from very young to sexually mature animals and numerous

egg capsules may be found in summer, but among the plants only the smaller, younger animals have been collected. Stocks are kept in the laboratory for three or four weeks before using experimentally so that uniformity in nutritive conditions may be approximated. They are fed three times a week with ground and washed beef liver, as described by Hyman ('20).

The work in this laboratory with planarians has demonstrated that in a stock of animals collected at one time, kept under as nearly as possible identical conditions of temperature, nutrition, water supply, etc., size is the best criterion of physiological condition, and particularly of physiological age, as indicated by susceptibility and respiratory rate, which can readily be applied to the living animals in the selection of material for experiment. Uninjured animals of the same size from such a stock show a high degree of uniformity in susceptibility to chemical and physical agents and in rate of respiration, as shown by the work of Child, Hyman, Behre, and Buchanan, and are more alike physiologically than material selected on any other basis thus far discovered. In these animals the amount of growth, whether rapid or slow, and consequently the size of the individual, is a far more exact measure of their physiological age, and so of their susceptibility and rate of respiration, than the length of time they have lived as individuals (Child, '15a, Chap. IV.; Hyman, '19 C).

Since the experiments recorded in this paper are all mass experiments—i.e., with numbers of individuals—the material for each experiment is selected on the basis of size. Such standardization of material is necessary for the attainment of definite results which can be predicted and controlled and it makes possible prediction and control to a high degree. In the course of the work experiments were performed with standardized material from general stocks collected and kept as described above, from stocks composed of animals hatched from eggs in the laboratory, and from stocks grown from cut pieces.

The experimental data presented below concern chiefly respiration, susceptibility, and head frequencies—i.e., the frequencies of occurrence of the various forms of head in the reconstitution of pieces in relation to level of body, length of piece, and physiological age of animals. Some experiments on modification and control of

head frequency by means of chemical agents have been performed, but are only briefly mentioned.

The data on respiration include comparative estimations of CO_2 production by colorimetric determination of pH with phenol-sulphone-phthalein as indicator and the indicator-buffer solutions made up by Hynson Westcott and Dunning as standards. In these experiments lots of as nearly as possible equal weights of animals or pieces to be compared are sealed in pyrex tubes of the same diameter as the standard tubes in equal volumes of indicator solution of the same concentration as the standard tubes and the change in color recorded at regular intervals and usually also to a definite pH. Some data on oxygen consumption determined by the Winkler method are also given. For these I am indebted to the kindness of Dr. Hyman.

In the experiments on susceptibility KNC has been chiefly used as agent, since it has been abundantly demonstrated that with proper precautions susceptibility to KNC can be used as a general comparative measure of physiological condition and particularly of rate of respiration in *Planaria*.[1] In the present study the susceptibility method has been used for two purposes: first, for demonstration of the axial gradients and the posterior zoöid by the differential susceptibility of different levels of the body; second, to demonstrate the changes in physiological condition of pieces following section. The general susceptibility gradients are shown in figures and the comparative susceptibilities of pieces by graphs. The method of graphing the data is described in connection with the data.

In the experiments on reconstitution the body posterior to the head is cut into a certain number of pieces—four, six, eight, sixteen—according to the experiment. Animals of the same size are used for each experiment and the pieces from each individual are cut as nearly as possible the same length, the more extreme irregularities being discarded. Consequently the corresponding pieces from different individuals represent as nearly as possible the same region of the body. All corresponding pieces—*i.e.*, those

[1] Child, '14a, c, '15a, chaps. III.–VII., '16, '19a, b; Hyman, '19a, b, c, '20, '22.

representing the same body region—are kept together in one container: The usual number of pieces in each lot is fifty, each taken, of course, from a different individual of the size used in that experiment. The pieces are allowed to undergo reconstitution and examination is made and results recorded after twelve to fourteen days, at which time reconstitution is so far advanced that no further change in the character of the form produced will occur. Since the forms produced fall into certain groups or types, as described in a following section, the results can be tabulated to show the frequency of each type in each lot. In this species, as in *P. dorotocephala,* the head is the most characteristic distinguishing feature of the different forms produced, except in very short pieces. The basis of tabulation is, therefore, form and structure of the anterior end, and the tables show the frequencies of the different forms and are called, for convenience, head frequency tables, this term having been used for similar data on *P. dorotocephala.* From the tabulated head frequencies graphs are constructed by a method described below, and in this way the head frequencies in different regions, in pieces of different lengths, and in animals of different size or physiological condition may be directly compared.

The relation of head frequency to region of body and length of piece is shown by comparing pieces of different lengths and from different body levels of animals of the same size, and therefore approximately of the same physiological age. The relation of head frequency to physiological age is determined by comparing the results obtained with pieces from animals of different lengths. In my experiments animals of two standard lengths have been chiefly used: full-grown animals, 11–13 mm. long, physiologically old, and with low rate of respiration; and young growing animals, 4–6 mm. long, with much higher rate of respiration. The smaller size is the smallest which can be conveniently used for such experiments because of the difficulty of cutting ·pieces of equal size in the smaller individuals. Some experiments have been performed with sizes intermediate between these two extremes, some with animals raised from eggs and some with animals raised from cut pieces.

THE AXIAL SUSCEPTIBILITY GRADIENTS.

The occurrence of definite axial susceptibility gradients in both animals and plants and their relation to metabolic rate has been discussed in various publications (*e.g.*, Child, '20*b*). Various lines of evidence show that susceptibility to a wide range of chemical agents in concentrations or intensities above the limit of tolerance or acclimation and below the limit at which death occurs immediately varies in general directly with metabolic rate, or more specifically with rate of respiration. Susceptibility gradients are characteristic features of physiological axes in both plants and animals and many facts indicate that such axes are primarily quantitative physiological gradients.

It has been found that in *P. dorotocephala* the head frequencies at different levels of the body show a definite relation to the polar susceptibility gradient, regions of high susceptibility being regions of high head frequency and vice versa. The observations on susceptibility in *P. lata* show that a similar relation exists in this species. In these observations KNC was used in most cases as agent, because it is known to be a powerful inhibitor of oxidations and has been extensively employed in the study of susceptibility, but many other agents of very different constitution—*e.g.*, $HgCl_2$, $CuSo_4$, acetic acid, various anesthetics, etc.—in proper concentration give essentially the same results. The procedure consists, first, in determining a concentration which kills slowly enough to permit the differences in susceptibility to appear clearly and which is not low enough to permit acclimation, and, second, in observing the progress of disintegration of the tissues of animals placed in the solution, in closed containers if necessary to avoid loss from volatilization. As death of any part occurs or approaches structural disintegration of the tissues takes place, and such disintegration appears first in certain regions and follows a definite course, so that certain body regions are completely disintegrated while others are still intact and moving.

The general course of disintegration in *P. lata* is shown in Figs. 7–10. The head and the posterior zoöid are most susceptible and disintegration progresses posteriorly from the head and at the same time involves a region anterior to the posterior zoöid and

posterior to the genital pore, the last region to disintegrate being that between the mouth and the genital pore. Commonly the lateral margins of the body disintegrate somewhat earlier than median regions at the same level, but in some individuals these transverse differences do not appear, and in some the median region apparently disintegrates earlier than the margins. There is not much difference between dorsal and ventral, but ventral regions usually disintegrate slightly in advance of dorsal (Fig. 11).
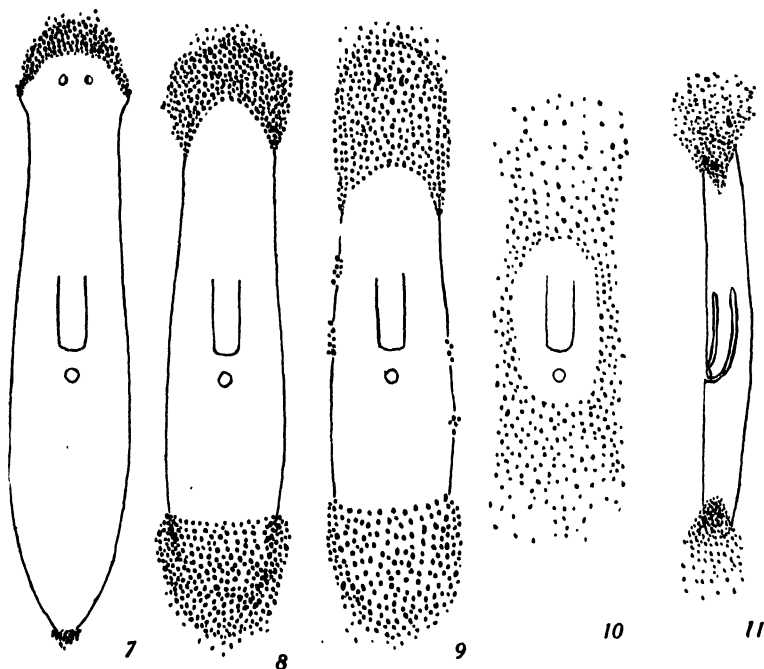
FIGS. 7–11. Susceptibility gradients in *P. lata,* as shown by KNC *m*/1000: Figs. 7–10, stages in progress of disintegration in dorsal view; Fig. 11, a stage of disintegration in lateral view to show difference between dorsal and ventral.

It should be noted that these statements concern primarily the body wall, but they appear to hold good for the parenchyma also. In well-fed animals the digestive tract is highly susceptible and, even though the cyanide must pass through the body wall to reach it, the digestive tract usually swells and disintegrates earlier than the body wall and often bursts through the latter at various points. In starved animals the digestive tract is much less susceptible and

in advanced starvation may remain intact longer than the body wall.

In general, the course of disintegration in this species is very similar to that in *P. dorotocephala,* but some minor differences appear. In long individuals of the latter, possessing more than one zoöid in the posterior region, the different zoöids usually show different susceptibilities (Child, '11c, '13b). The early disintegration of the region anterior to the posterior zoöid in *P. lata* may indicate the presence here of another zoöid at a very early stage; in other words, some degree of physiological isolation with increase of metabolic activity may exist anterior to the level of fission and yet be insufficient to permit fission at this level.

In *P. dorotocephala* the margins of the body always disintegrate in KNC before median regions, while in *P. lata* this may or may not be the case. These differences are apparently associated with the specialization of the margins as motor organs, and particularly as organs of secretion of slime. There are many glands in this region and the alkaline cyanide stimulates these to increased activity. Often the separate glands disintegrate before other parts of the margins, particularly in *P. dorotocephala.* Apparently the margins of *P. lata* are less highly specialized in this way, for the glands appear less distinctly as regions of disintegration and there is less motor activity of the margins. In neutral or acid cyanide and in other acid agents the glandular activity is apparently not increased and motor activity is decreased, and in such solutions the susceptibility of the margins, even in *P. dorotocephala,* is usually less than that of median regions.

In *P. dorotocephala* the dorsal body wall disintegrates earlier than the ventral in alkaline cyanide. The dorsal wall is thinner than the ventral and shows many localized regions of disintegration, apparently glandular. In acid agents ventral regions are usually more susceptible. In *P. lata* the specialization of the dorsal surface is apparently less, so that even in alkaline agents the difference between dorsal and ventral is slight and the ventral surface is usually the more susceptible.

In the early developmental stages of turbellaria the median ventral region is more susceptible than lateral and dorsal regions (Child, unpublished), and in full-grown planarians the new tissue

develops more rapidly from the median ventral region than from other parts of a cut surface. This fact suggests that internally the median ventral region still possesses the highest metabolic rate. Apparently the primary symmetry gradients undergo more or less alteration in the body wall of some species, and the susceptibility data indicate that such alteration is greater in *P. dorotocephala* than in *P. lata.* The latter species seems to retain more nearly the characteristics of earlier stages.

Young animals are always more susceptible than old and the differences in susceptibility at different levels of the body are less in the young. In fed animals susceptibility decreases from the time of hatching to maturity and in this respect parallels rate of respiration (Child, '19*a*; Hyman, '19*c*). In full-grown animals the time from the beginning to the end of disintegration in *m*/1,000 KNC at 20° C. is eight to ten hours; in young animals it is much less, increasing with advancing age. High temperature increases, low temperature decreases susceptibility. Starvation increases susceptibility and also decreases the differences at different levels. In all these respects the two species are alike.

THE FORMS RESULTING FROM RECONSTITUTION.

. As in *P. dorotocephala,* the results of reconstitution differ in definite and orderly ways according to length of piece and region of body from which it is taken. Some of these differences, such as the level at which the new pharynx appears, length of prepharyngeal and postpharyngeal regions, are merely temporary features of the earlier stages of reconstitution, and are later largely or completely obliterated by differential growth of prepharyngeal and postpharyngeal regions, particularly if the new individuals are fed, until finally all normal or nearly normal individuals attain approximately the same proportions.

The most conspicuous differences in the results of reconstitution concern the head and these differences are, with certain rare exceptions, permanent. Isolated pieces do not always develop anterior ends like that of the normal animal in nature, but abnormal forms occur which constitute a continuous series with some secondary modifications from the normal head to a completely headless con-

dition.   The members of this series fall readily into the same types or groups as in the case of *P. dorotocephala* (Child, '11a, '21), viz., normal, teratophthalmic, teratomorphic, anophthalmic, acephalic.   The normal head is like the head of Fig. 1.   The teratophthalmic head is normal in outline, but the eyes are more or less approximated to the median line and the pigment spots are often partially united or even fused (Figs. 12, 13).   In the ˙teratomorphic head there is a single, or apparently single, median eye and the anterior region between the cephalic lobes is incompletely developed, so that the lobes appear more or less anteriorly or fused at the median line (Figs. 14, 15).   The anophthalmic head is merely an outgrowth of tissue without eyes (Figs. 16, 17), and in
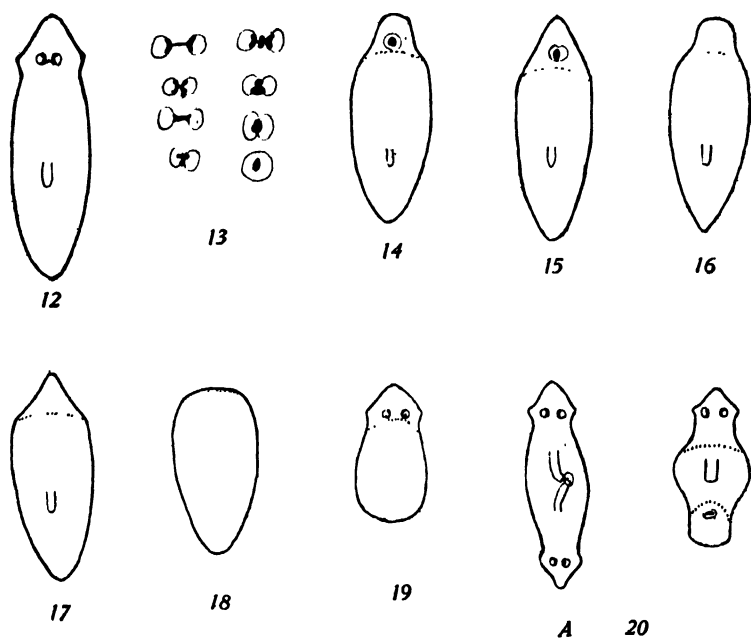
FIGS. 12–20. Forms resulting from reconstitution: Figs. 12, 13, teratophthalmic heads and eyes: Figs. 14, 15, teratomorphic heads; Figs. 16, 17, anophthalmic heads; Fig. 18, acephalic form; Fig. 19, tailless form; Fig. 20, biaxial heads.

the acephalic form the anterior end simply heals over without outgrowth (Fig. 18).   As regards degree of development, the cephalic ganglia of these forms also constitute a continuous series from normal ganglia in the normal head to a rudimentary ganglion in

the anophthalmic, and no ganglia in the acephalic form (Child and McKie, '11). In this species, as in *P. dorotocephala*, no other head forms have been observed except secondary modifications of some one of these forms in acclimation to, or recovery from, inhibiting agents (Child, '21) and inequalities or asymmetries in position of eyes resulting from oblique section or other incidental conditions and usually temporary. The frequency of occurrence of these various forms in any lot of pieces is the head frequency of the lot, and the following experiments on reconstitution are chiefly concerned with the relations of head frequency to length of piece, level of body, and size of animal from which pieces are taken.

The results of reconstitution in very short pieces require mention. Forms with heads, usually normal, but without posterior ends, "tailless forms" (Fig. 19) and "biaxial heads" (Fig. 20), appear rarely in sixths of large animals, more frequently in eighths, and their frequency increases with decreasing length of piece to the limit of length at which wound closure and reconstitution fail to occur. Under ordinary conditions these forms are much more frequent and appear in longer fractions of the body in this species than in *P. dorotocephala*. In the latter species they have never been seen in sixths or eighths, except rarely in eighths from small young animals, and they are rare even in sixteenths and twentieths of large animals.

HEAD FREQUENCIES IN RELATION TO LENGTH OF PIECE, LEVEL OF BODY, AND PHYSIOLOGICAL AGE OF ANIMAL.

The data presented in this section include head frequencies of fourths, sixths, eighths, and sixteenths of full-grown animals 11–13 mm. in length and fourths and sixths of young animals 4–6 mm. in length. In the series of longer pieces the death rate is negligible, but in the sixths of young and the sixteenths of old animals it becomes high enough to lessen considerably the value of the data on head frequencies, and in pieces shorter than these it is still higher, so that determination of head frequencies becomes impossible in such pieces.

This increasing death rate with decreasing length of piece is certainly to a considerable extent a consequence of increasing area

of cut surface in relation to size of piece. The deaths are practically limited to the first day or two following section. Pieces that survive this period live, with rare exceptions, to the end of the experiment and undergo some degree of reconstitution. In such very short pieces of *P. dorotocephala* the contraction of the cut surface often brings about rupture elsewhere, and contraction at this point produces further rupture in other regions and the piece gradually breaks up. In *P. lata* also the contraction following

TABLE I.

HEAD FREQUENCIES OF FULL-GROWN *P. lata* (11–13 MM.) IN RELATION TO
LENGTH OF PIECE AND LEVEL OF BODY.

Length of Pieces.	Number of Animals.	Body Level.	Normal.	Teratophthalmic.	Teratomorphic.	Anophthalmic.	Acephalic.	Dead.	Biaxial Heads.
Fourths.......	210	A	99	—	—	—	1	—	—
		B	47	33	—	6	10	4	—
		· C	77	16	—	3	3	1	—
		D	96	1	—	1	1	1	—
Sixths........	250	A	98	1	—	—	·—	1	—
		B	53	30	1	5	9	2	.4
		C	48	33	2	6	8	3	—
		D	43	17	2	8	29	1	—
		E	83	11	1	1	2	2	—
		F	98	1	—	1	—	—	—
Eighths.......	850	A	95	1	—	—	1	3	—
		B	73	16	.5	.5	6	4	.6
		C	46	27	1	3	16	7	.8
		D	37	27	1	8	22	5	.7
		E	39	17	2	6	32	3	1
		F	66	11	2	2	16	3	1.6
		G	85	6	—	1	4	. 4	.9
		H	96	2	—	—	—	2	—
Sixteenths.....	100	A	72	1	—	—	—	27	—
		B	61	4	—	1	—	34	1
		C	46	2	—	2	4	46	1
		D	47	8	—	2	8	35	3
		E	39	13	—	1	13	34	2
		F	17	13	—	10	22	38	2
		G	22	9	—	7	21	41	1
		H	29	6	—	2	24	39	4
		I	37	5	—	3	26	29	2
		J	26	7	—	9	28	30	3
		K	34	8	—	11	20	27	6
		L	42	8	1	6	10	37	6
		M	45	13	—	5	5	32	6
		N	47	5	—	4	10	34	6
		O	77	5	—	2	7	9	4
		P	88	5	—	—	1	6	—

section is the chief factor in determining these early deaths in short pieces. In the experiments presented here the pieces dying in this manner are included in the totals in calculating percentages and in graphing, but in certain lines of future investigation it will probably be desirable to exclude these early deaths in determining total head frequencies.

The head-frequency data are tabulated in percentages, the full-grown animals in Table I., the young animals in Table II. From

TABLE II.

HEAD FREQUENCIES OF YOUNG *P. lata* (4–6 MM.) IN RELATION TO LENGTH OF PIECE AND LEVEL OF BODY.

Length of Pieces.	Number of Animals.	Body Level.	Normal.	Terato-phthalmic.	Terato-morphic.	Ano-phthalmic.	Aceph-alic.	Dead.	Biaxial Heads.
Fourths.......	150	A	90	5	—	1	1	3	—
		B	51	17	—	1	15	16	—
		C	72	15	—	—	7	6	.7
		D	85	3	—	1	3	8	—
Sixths........	150	A	91	3	—	—	1	5	—
		B	61	23	1	1	5	—	—
		C	48	34	—	2	9	7	—
		D	48	23	—	1	17	11	.7
		E	69	6	—	1	5	20	.7
•		F	95	4	—	—	1	—	—

the tabulated data graphs are plotted by the method of assigning numerical values to the different head forms as follows: Normal heads, 5; teratophthalmic, 4; teratomorphic, 3; anophthalmic, 2; acephalic, 1; dead, 0. To obtain a head-frequency value for a given lot of pieces, the number of pieces or the percentage of each head form is multiplied by the numerical value of that form and the sum of these products for the particular lot is divided by the number of pieces in the lot, or, if the data are in percentages, by one hundred. The results are the same whether actual number of pieces or percentages are used. These values plotted as ordinates against the successive levels of section, *A, B, C,* etc., from the heads of the original animals posteriorly as abscissæ, give curves which permit direct comparison of the head frequencies of pieces of different lengths and from different levels.

FIG. 21.   Head frequencies in relation to length of piece and level of body. *P. lata:* fourths, unbroken line, 110 animals; sixths, long dashes, 250 animals; eighths, short dashes, 850 animals; sixteenths, alternating long and short dashes, 100 animals. *P. dorotocephala:* fourths, dotted line, 110 animals; sixths, alternating dots and long dashes, 110 animals; eighths, alternating dots and short dashes, 70 animals; sixteenths, two dots alternating with short dashes, 60 animals. On the outline of *P. lata* below the graph the levels of section of pieces of different length are indicated: *A–D,* large capitals, fourths; *A–F,* small capitals, sixths; *a–h,* large lower case, eighths; *a–p,* small lower case, sixteenths. The levels of corresponding pieces of *P. dorotocephala* are in part slightly different from those indicated in the diagram since in this species the posterior zoöid is much longer and the pharynx therefore nearer the anterior end in large animals than in *P. lata.*

In the graph, Fig. 21, the data for full-grown animals given in Table I. are plotted, together with head-frequency curves from similar experiments with *P. dorotocephala*. In Fig. 22 the data for young worms from Table II. are graphed in comparison with corresponding data of *P. dorotocephala*.[2] As regards region of body, the data show, both for full-grown and for young animals, that head frequency decreases from the most anterior level of.section posteriorly and then increases again with approach to the level of the posteriod zoöid, until at the most posterior level of section it is as high as at the most anterior level, except when lowered by early deaths.

Second, as regards length of piece, the data show for old animals that decrease in head frequency from anterior to posterior region of the first zoöid and increase at levels further posterior becomes greater as length of piece becomes less. The shorter the pieces, the steeper the downward and upward slopes of the curves in Fig. 21. At the most anterior and most posterior levels of the body fourths, sixths, and eighths are practically alike in head frequency, sixteenths somewhat lower anteriorly, but the differences in level of the lowest points of these curves is considerable. The irregularities in the curve of sixteenths are due to the differences in length of the pieces, the variations being, of course, relatively much greater in these very short pieces than in longer pieces. In the young worms (Fig. 22) the differences in steepness in relation to length of piece do not appear in the only data available, those for fourths and sixths, but the sixths show a somewhat lower head frequency than the fourths, except at the posterior end, where it is the same in both.[3]

[2] The curves of head frequency of *P. dorotocephala* are plotted from data obtained from various sources: fourths, sixths and eighths of old animals and fourths and sixths of young animals from Child ('11b, '16, '20a), one series of eighths of old animals from Miss M. A. Hinrichs and a complete series of my own for both old and young. All of these data are in general agreement as regards relation of head frequency to length of piece, level of body and physiological age.

[3] It may be noted that the data for young worms are made up in part from animals raised in the laboratory from eggs and in part from animals of the same size similarly raised from cut pieces. The pieces of animals from eggs showed a somewhat higher death rate and therefore a somewhat lower head frequency than those from cut pieces, but the differences were not great.
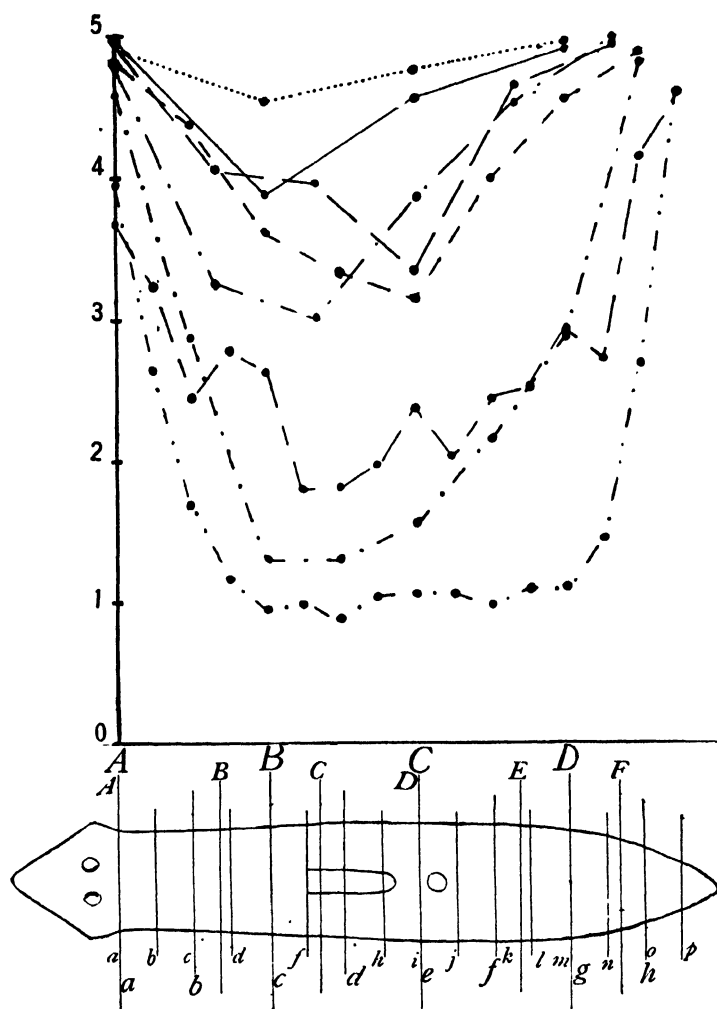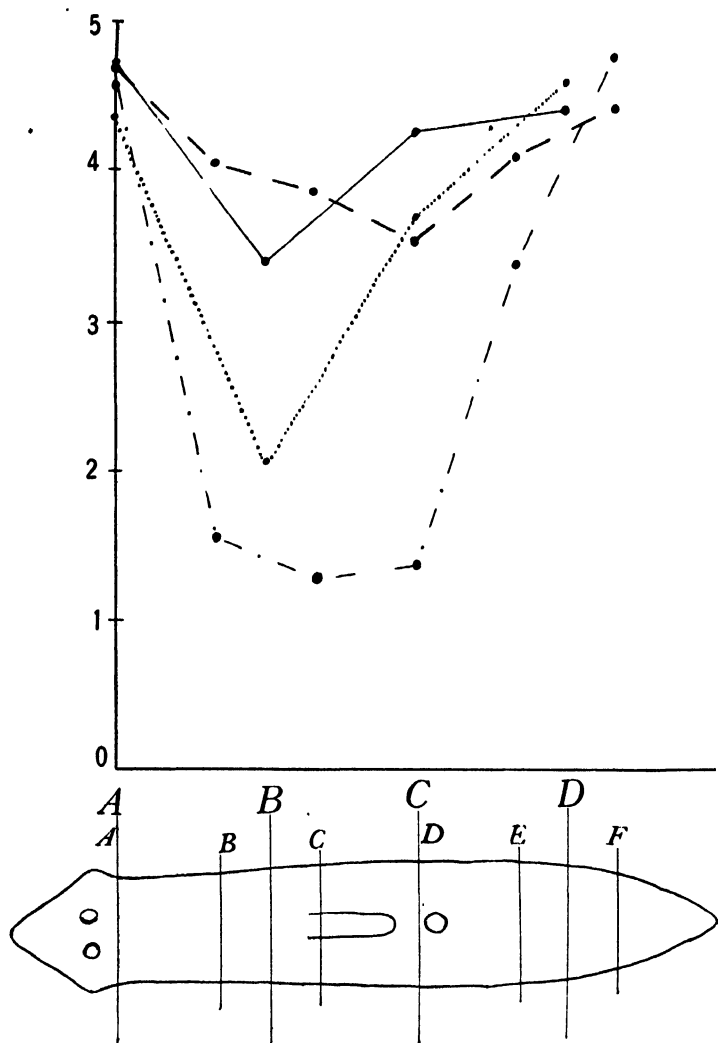
FIG. 22. Head frequencies in relation to length of piece and level of body in young animals. *P. lata:* fourths, unbroken line, 150 animals; sixths, dashes, 150 animals. *P. dorotocephala:* fourths, dotted line, 60 animals; sixths, dots and dashes, 100 animals. The diagrammatic outline below graph indicates levels of section: *A–D*, large capitals, fourths; *A–F*, small capitals, sixths.

Since the number of animals obtained from eggs was limited, it was not possible to determine whether these differences were characteristic or merely the result of slight differences in experimental conditions. For the present, therefore, it has seemed best to combine these data in tabulation and graphing.

Comparison of the curves for *P. lata* with those for *P. doroto-cephala* in Figs. 21 and 22 shows that the changes in head frequency with length of piece and level of body are in general of the same sort, but that their range is very much greater in *P. dorotocephala* than in *P. lata*. In full-grown animals (Fig. 21) the head frequencies of fourths of *P. dorotocephala* are somewhat higher than, those of sixths about equal to, and those of eighths and sixteenths far lower than the head frequencies of corresponding pieces of *P. lata* from the posterior regions of the first zoöid, ·while at anterior and posterior ends of the body the differences between the two species are slight. In the young animals (Fig. 22) fourths and sixths from the posterior region of the first zoöid of *P. dorotocephala* are far below fourths and sixths of *P. lata* from the same region, while at the anterior end of the body the differences are much less, though greater than in old animals.

The graph, Fig. 23, is a comparison of the curves of fourths and sixths of full-grown and young individuals of *P. lata*. This graph shows that the head frequencies in young animals are slightly lower than in corresponding pieces of old animals, but this age difference is much less than in *P. dorotocephala*. Comparison of Figs. 21 and 22 shows that the curves of fourths and sixths of young *P. dorotocephala* are much steeper and fall much lower than those of fourths and sixths of old animals.

Tables I. and II. show the frequencies of biaxial heads, but not of tailless forms. Unfortunately tailless forms were not recorded as such in the earlier experiments: It may be stated, however, that they do not appear in the longer pieces, that their frequency increases with decreasing length of piece, and that, so far as data are at hand, they show no definite relation to level of body. As ·regards the frequencies of biaxial heads, Tables I. and II. show that they do not occur in the longer pieces, that their frequency increases with decreasing length of piece, and is apparently somewhat greater near the level at which fission occurs than elsewhere.

Experimental Alteration of Head Frequency.

In *P. dorotocephala* the head frequencies have been altered experimentally by many different chemical and physical factors (Behre, '18; Buchanan, '22; Child, '16, '20a). Moreover, it has

been found that head frequencies may be altered in opposite direc-
tions in pieces from different levels of the same animals by the
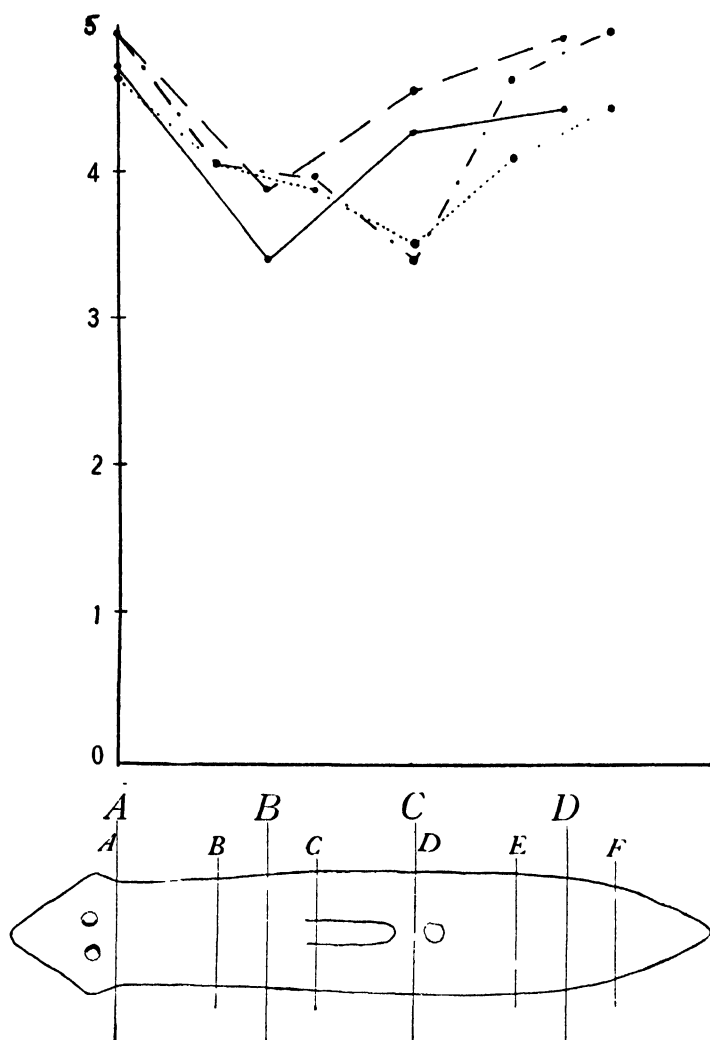
FIG. 23. Head frequencies of fourths and sixths in old and young individu-
als of *P. lata:* fourths, young, unbroken line; sixths, young, dotted line;
fourths, old, long dashes; sixths, old, dots and dashes. The curves in this
graph are taken from Figs. 21 and 22.

same concentration of a single agent (Child, '16; Buchanan, '22).
In pieces which normally show a high head frequency it may be
decreased and in pieces which normally show a low head frequency

it may be increased by the same concentration of cyanide or anesthetic. On the basis of these and many other facts concerning the physiology of reconstitution a theory of head frequency has been advanced (Child, '14a, '14d, '16) which is confirmed by later work (Buchanan, '22). This theory, which is considered more fully below (p. 56), maintains that head frequency in any particular case is determined primarily by the relation between two opposing factors: the one the rate of metabolism in the cells at the cut surface which form the head, the other the rate of metabolism in other parts of the piece. The higher the first in relation to the second, the higher the head frequency; the higher the second in relation to the first, the lower the head frequency. The differential susceptibility of the cells at the cut surface and the other parts of the piece makes it possible through differential inhibition, differential acclimation, and recovery (Child, '20b, '21) to alter the relation between the two factors in both directions.

The correlative factor retarding or inhibiting head formation is apparently in part or wholly a matter of nervous stimulation of the cells not directly concerned in head formation. It is most effective during the first few hours after section, when increased CO_2 production, oxygen consumption and susceptibility all show that the pieces are stimulated. In *P. dorotocephala* this stimulation is inhibited and head frequency increased by anesthetics such as ether and chloroform used during a few hours following section (Buchanan, '22), but a day or two later such anesthetics in the same concentrations bring about no increase (Buchanan, unpublished).

Concerning the experiments on *P. lata,* it may be noted, first, that the two factors are concerned in this species, and, second, that apparently nervous stimulation is less effective in decreasing and nervous inhibition in increasing head frequency than in *P. dorotocephala.* Head frequency can be altered in both directions in *P. lata,* but so far as experiments have gone, it appears that general protoplasmic depressants, such as acids; are highly effective, while anesthetics in the stricter sense have little or no effect. These facts are in accord with observations on the living animal, which indicate that the nervous organization is considerably lower than in *P. dorotocephala.*

Another fact pointing in the same direction is that the original polarity can be more readily obliterated and biaxial heads produced by chemical agents than in *P. dorotocephala*. In certain concentrations of acids, for example, the frequency of biaxial heads is much increased.

THE PHYSIOLOGICAL CONDITION OF PIECES FOLLOWING SECTION.

Section of the body results in exposure of a cut surface which gradually contracts and within a few hours cell division and growth begin, giving rise to new embryonic tissue. It has been shown for *P. dorotocephala* that increase in rate of respiration and in susceptibility is slight or inappreciable in fourths or longer pieces, while in sixths and shorter pieces it is marked and increases as length of piece decreases, and also increases from anterior to posterior levels of the first zoöid and decreases again in the posterior zoöids (Child, '14a; Robbins and Child, '20; Buchanan, '22; Hyman, '22). Since these changes show definite relations to the polar gradient and are factors in determining head frequency (Child, '14d, '16; Buchanan, '22), it is of interest to determine whether similar changes occur and influence head frequency in *P. lata*.

Changes in Susceptibility Following Section.—The data are most readily presented as graphs, plotted by the method used in earlier work (Child, '15a, p. 81). This method is briefly as follows: Five stages in the progress of disintegration in KNC or some other agent from intact animals or pieces to completely disintegrated are more or less arbitrarily distinguished and these are given, respectively, the numerical values, 40, 30, 20, 10, 0. In determinations of susceptibility a certain number—*e.g.*, ten—of animals or pieces is placed in the solution used and the number or individuals or pieces in each stage is recorded at hourly or half-hourly intervals. The ordinate of the susceptibility curve for any time is the sum of the products obtained by multiplying the number for each stage by the numerical value of that stage, divided by the number of animals or pieces in the lot. These ordinates are plotted against times in hours as abscissæ.

In the experiments on pieces the susceptibility of the fourths, sixths, and eighths was determined separately for each level of the

body; but since the pieces from different levels showed no great differences in susceptibility, the data for all the different levels were brought together in a single curve for each length of piece. Consequently each curve of pieces in the graph, Fig. 24, represents pieces from all levels—*i.e.*, the whole body except the head cut into fourths, sixths, or eighths.   KNC *m*/1,000 was used as agent.
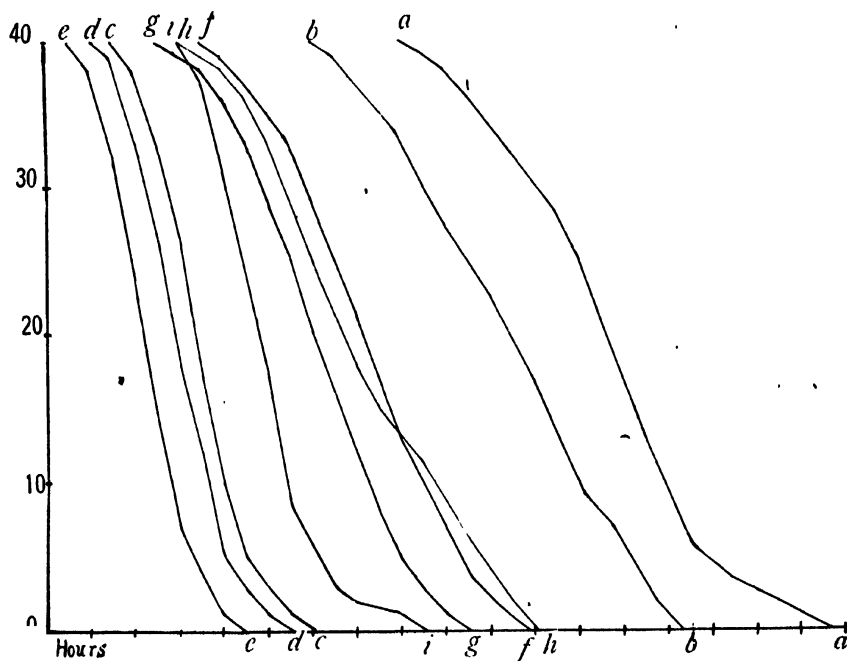
FIG. 24. Graph showing changes in susceptibility following section. Each curve except *aa* and *bb* represents all pieces from 50 animals, *aa*, 50 intact animals, *bb*, 20 headless animals. Further explanation in text.

Fig. 24 shows that susceptibility is greatly increased by section. Uninjured, full-grown animals (*aa*) show the lowest susceptibility of all.   Removal of the head increases susceptibility (*bb*).   In fourths (*cc*), sixths (*dd*), and eighths (*ee*), immediately after section, susceptibility is greatly increased, and it will be noted that it is highest in eighths, somewhat lower in sixths, and lowest in fourths.

During the first fifteen to twenty hours after section the susceptibility of pieces decreases, then remains nearly stationary for

a day or two, and then gradually rises as reconstitution progresses. Curve *ff* shows the susceptibility of fourths left in water fifteen to eighteen hours after section and then placed in KNC; curve *gg*, that of sixths under the same conditions; curve *hh*, that of eighths after forty hours in water. And, finally, curve *ii* shows the susceptibility of young growing animals 5 mm. long raised from pieces—*i.e.*, approximately the susceptibility attained by fourths after reconstitution is completed—that of sixths and eighths being somewhat higher.

Some part of this increase of susceptibility after section is undoubtedly due to the presence of one (*bb*) or two (*cc, dd, ee*) cut surfaces. Other experiments for which curves have been plotted, but which are not shown here, demonstrate that pieces with oblique and therefore larger cut surfaces are more susceptible than pieces with transverse surfaces.

The experiments on susceptibility show one other point of importance which does not directly appear in Fig. 24 and which would require a number of graphs for full presentation. It was stated above that pieces of the same length from different levels show approximately the same susceptibility. In the section on susceptibility gradients it was shown that susceptibility decreases from the anterior to the posterior end of the first zoöid and increases again with approach to the level of the posterior zoöid. Even if the susceptibility of pieces from all levels were approximately the same immediately after section, it would be evident that the increase in susceptibility must have been much greater in pieces from posterior than in those from anterior levels of the first zoöid and from the posterior zoöid. It follows from this fact that susceptibility is not simply a matter of the presence of cut surfaces, but depends in part upon level of the body from which pieces are taken.

Comparison of these changes in susceptibility following section with those in *P. dorotocephala*[4] brings to light some interesting physiological differences between the two species. In the first place, removal of the head does not appreciably increase susceptibility in *P. dorotocephala*. In fourths it is increased only slightly,

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[4] See Child, '14a. His results have been repeatedly confirmed by myself and by other students in laboratory experiments.

in sixths somewhat more, and in eighths still more, but in all cases much less than in *P. lata.* Moreover, during the first twenty-four hours after section it usually decreases almost or quite to the same level as that of whole animals. In short, the presence of one or even two cut surfaces has in itself little or no effect in increasing susceptibility in *P. dorotocephala.* Differences in body level are far more important, particularly in the shorter pieces. Even in sixths or less the increase is slight in anterior pieces, becomes greater toward the posterior end of the first zoöid, and is again slight in the posterior zoöid.

Evidently the increase in susceptibility in relation to cut surfaces and the differential increase at different levels of the body depend, at least in part, on different factors. The former, which seems to be essentially a cellular wound reaction, followed by cell division and growth, is the more conspicuous feature in *P. lata.* The differential increase, on the other hand, is more conspicuous in *P. dorotocephala,* but is present also in *P. lata,* and appears to be a stimulation of the piece as a whole. Various facts, such as its short duration and its inhibition by anesthetics (Buchanan, '22), indicate that it is nervous in character. Moreover, it is of interest to note that the differential increase varies inversely as susceptibility at different levels of whole animals and inversely as head frequency at different levels, while the increase in relation to cut surfaces varies directly with area of cut surface in relation to size of piece.

Changes in Rate of CO_2 Production Following Section.—Colorimetric estimations of CO_2 production confirm the data on susceptibility as indicative of changes in rate of respiration. In each of these experiments five 12–13-mm. headless animals entire were compared with five headless animals cut into eighths. The starting point was pH 7.9, and the pH was recorded at regular intervals, and the time required to reach pH 7.3 was also determined. After the experiment both lots were weighed, and in all cases the weight of the pieces was less by some 20 to 50 per cent. than the weight of headless animals, because some loss of intestinal contents, fluids, or even cells occurs when pieces are cut. In all cases, however, the rate of decrease of pH of the pieces was equal to or

higher than that of the entire headless animals.   In Table III. the length of time required for change from pH 7.9 to pH 7.3 is given in hours and minutes for ten milligrams of headless animals and þieces, as calculated from the actual weights and times.   This method of presentation is open to certain objections, but has the advantage of brevity and clearness.   Of the nine experiments in Table III. five were with headless animals and eighths immediately after section, four with headless and eighths twenty-four hours

TABLE III.

TIME IN HOURS AND MINUTES REQUIRED FOR CHANGE FROM pH 7.9 TO 7.3
CALCULATED FOR 10 MG. FROM ACTUAL WEIGHT AND TIME.

| Headless. | Eighths. | |
	Immediately after Section.	24 Hours a. s.
10:36	4:10	——
8:49	4:57	——
10:30	——	4:10
12:20	3:42	——
12:40	——	9:20
6:12	——	5:12
10:40	· 4:53	——
8:30	6:34	——
7:22	——	4:44
Mean, 9:48	4:51	5:51

after section.   In every case the time is much less for the pieces, but is somewhat greater after twenty-four hours than immediately after section.   These data confirm the susceptibility data in showing that rate of respiration in eighths immediately after section is greatly increased over that of headless controls, and that after twenty-four hours it is somewhat lower, but still far above that of the controls.

   Changes in Oxygen Consumption Following Section.—Table IV. gives the results of determinations of oxygen consumption made by Dr. Hyman.   In the first experiment wholes, headless, and pieces are compared, in the others only headless and pieces, the headless animals being used because they show less motor activity than wholes.   The pieces include both sixths and eighths from all levels posterior to the head.   Table IV. shows that oxygen consumption is greatly increased in pieces immediately after section, as compared with headless animals, and in the first experiment in headless animals as compared with wholes.   Twenty-four hours after section the oxygen consumption is lower in some cases, higher

in others, than immediately after section, and increase is more frequent in the pieces than in headless animals. Pieces of *P. dorotocephala* also show a similar increase in oxygen consumption

TABLE IV.

OXYGEN CONSUMPTION OF *P. lata:* WHOLE ANIMALS, HEADLESS, AND SIXTH AND EIGHTH PIECES IMMEDIATELY AND TWENTY-FOUR HOURS AFTER SECTION. CALCULATED IN CUBIC CENTIMETERS OF OXYGEN CONSUMED PER GRAM IN FOUR HOURS. CALCULATIONS AT 20° C.

Experiment.	Wholes.	Headless.		Pieces.	
		Immediate.	24 Hours.	Immediate.	24 Hours.
1........	1.31	1.40	1.30	1.93	1.68
				2.11	1.78
2........	——	1.06	.99	1.44	Lost.
3........	——	1.34	1.47	1.60	1.83
4........	——	1.26	1.11	1.51	1.71
5........	——	1.00	1.20	1.59	1.79
				1.73	2.18

in some cases (Hyman, '22). In this respect the data on oxygen consumption apparently disagree in part with those on CO_2 production and susceptibility. The reasons for this apparent disagreement are not as yet certainly known, but it seems probable that nutritive condition and growth at the cut surfaces which is appreciable within twenty-four hours are the factors chiefly concerned.

TIME OF HEAD DETERMINATION.

It has been shown that in *P. dorotocephala* the head frequency characteristic of a certain length of piece at a certain level is determined within a few hours after section to such an extent that it is only slightly or not at all altered by decreasing the length of piece after that time (Child, '14d). The experiment to test this point gives essentially the same results in *P. lata,* except that the differences in head frequency in long and short pieces are less than in *P. dorotocephala*. Fig. 25 shows the pieces used in the experiment. A large number of long pieces are cut with anterior ends

at level *aa* (Fig. 25), and at intervals the anterior ends of a certain number of them—*e.g.*, fifty—are cut off as short pieces at the level *bb* and their head frequencies recorded after reconstitution. Omit-
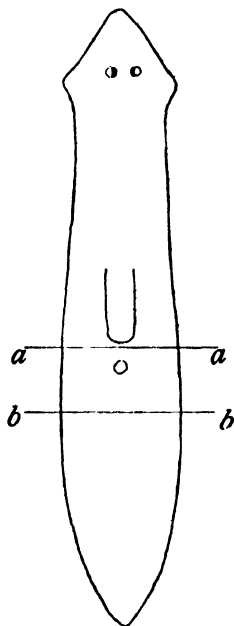
FIG. 25. Outline indicating levels of section, *aa* and *bb*, in experiment on head determination.

ting tables and graphs, the most important results obtained are as follows: In the short pieces isolated at once in the usual manner the chief head frequencies are: normal, 40 per cent.; teratophthalmic, 17 per cent.; acephalic, 30 per cent.   In short pieces cut from anterior ends of long pieces after five hours head frequencies are: normal, 80 per cent.; teratophthalmic, 6 per cent.; acephalic, 2 per cent.   In short pieces cut in the same way after twenty-three hours 100 per cent. normal heads appeared.

This experiment shows that under ordinary conditions factors determining head frequency are to some extent effective during the first few hours after section, for during this period the alteration of head frequency characteristic of long pieces by great decrease in length of piece becomes progressively less.   This, of course, does not mean that head frequency can not be altered in other ways after this time; it merely means that conditions deter-

mining head frequency are to some extent established during this time. Since this is the period of greatest stimulation of pieces, since that stimulation is greater in shorter than in longer pieces and in posterior than in anterior pieces of an individual or zoöid, and since decrease of that stimulation during the first few hours after section increases head frequency, it appears highly probable that the differential stimulation of pieces after section is a factor in determining that under ordinary conditions head frequency is lower in shorter than in longer and in posterior than in anterior pieces of individual or zoöid.

RATE OF GROWTH AND DIFFERENTIATION AND REGIONAL DIFFERENTIALS IN DEVELOPMENT AT ANTERIOR CUT SURFACES.

Only a brief statement of general results along these lines is given at the present time. The growth reaction at anterior cut surfaces is much more rapid in *P. lata* than in *P. dorotocephala*. In the former the strong contraction following section has very largely disappeared and a distinct outgrowth of new tissue is present over the whole surface twenty-four hours after section (Fig. 26), while in the latter the cut surface is still strongly contracted and there is less than half as much new tissue (Fig. 27). On posterior surfaces the differences between the two species are less marked (Figs. 26, 27). Two days after section the differ-

26   27

FIGS. 26, 27.   New tissue twenty-four hours after section: Fig. 26, *P. lata;* Fig. 27, *P. dorotocephala.*

ences between the species remain about the same, but during the third day the rate of growth in *P. dorotocephala* becomes more rapid, as compared with that in *P. lata,* and on the fourth day the amounts of new tissue are apparently about the same. Evidently the initiation and acceleration of growth at the cut surface occurs

earlier in *P. lata* than in *P. dorotocephala*. This difference probably accounts, at least in part, for the fact that susceptibility and rate of respiration in pieces of *P. lata* remain considerably higher after section than in whole animals, while in *P. dorotocephala* the decrease within twenty-four hours after section is greater, often to the level of whole animals.

The rate of growth and of differentiation of the head, as determined by the time at which the eyes become visible, is approximately the same in anterior regions· and in the posterior zoöid in both species, and in both it is more rapid in these regions than at posterior levels of the first zoöid. It also decreases and the differences in rate at different levels increase with decreasing length of piece. In other words, curves of rate of differentiation of head plotted from repeated observations of developing pieces resemble in their relations to body level and length of piece the head-frequency curves. Comparison of the two species shows, however, that in the shorter pieces and the more posterior levels of the first zoöid the rate of differentiation is slightly lower in *P. lata* than in *P. dorotocephala*. That is to say, in *P. lata* the head frequency in such pieces is higher, but the rate of differentiation of the head is lower than in *P. dorotocephala*.

As regards the portion of body posterior to the head which is formed by new tissue, there is little difference at different levels of section in longer pieces, but with decreasing length of piece this differential appears. In eighths, for example, from levels near the anterior end of the animal and near or in the posterior zoöid, the eyes develop at or slightly anterior to the boundary between new and old tissue (Figs. 28, 29), while in pieces from the poste-

28   29   30

FIGS. 28–30. Regions of body formed by anterior new tissue at different levels in eighths of *P. lata:* Fig. 28, anterior region of first zoöid; Fig. 29, posterior zoöid; Fig. 30, posterior region of first zoöid.

rior region of the first zoöid—*i.e.*, about the level of the pharynx—
a considerable portion of the body posterior to the eyes develops
from new tissue (Fig. 30). A similar regional differential ap-
pears in shorter pieces in *P. dorotocephala* and *P. maculata*, but
the amount of difference differs somewhat in the different species.
In *P. foremannii* and other species which possess no posterior
zoöid the regional differential continues to change in the same
direction to the posterior end of the body—*i.e.*, the more posterior
the piece, the longer the portion of the body formed from new
tissue at the anterior end (Morgan, '01).

This regional differential is evidently determined by a complex
of factors—*e.g.*, rate of growth of new tissue, size of new head,
degree of inhibition in its development by stimulation of the piece,
rapidity of reorganization of old parts, and perhaps others. The
chief point of interest at present, however, is the fact that this
regional differential shows the same relation to body level and
length of piece as does head frequency. Its relation to the polar
axial gradient is therefore evident. Moreover, experiments show
that this differential can be altered and controlled by the same
factors by which head frequency is altered and controlled.

DISCUSSION.

*Reconstitution in Relation to Body Level, Length of Piece, and
Physiological Age.*—It is evident that the axial susceptibility gra-
dient is an indicator of fundamental physiological differences along
the axis and many facts indicate that such differences are pri-
marily quantitative rather than qualitative. Head frequency, dif-
ferential increase in susceptibility following section, rate of growth
of new tissue at the anterior end, and the portion of the body
posterior to the eyes which is formed by new tissue all show a
gradation according to body level and therefore a definite relation
to the susceptibility gradient. This is true for *P. dorotocephala*
as well as for *P. lata*. That the physiological factors which deter-
mine these graded differences in reaction to section and in the
processes of reconstitution are fundamentally quantitative, not
qualitative, is indicated by the fact that there is no evidence of
fixity or specificity in their relation to body level. All the features
of reconstitution characteristic of a given body level under normal

or standard external conditions can be altered to those character-istic of other levels by changes in external conditions which are primarily non-specific and quantitative in physiological effect. This has been shown for *P. dorotocephala* in many ways and is also true for *P. lata*, though only a part of the evidence appears in the present paper. It has also been shown for *P. dorotocephala* that the susceptibility gradient is an indicator of a corresponding gradient in rate of respiration (Robbins and Child, '20; Hyman, '22), and the data on the changes in susceptibility and rate of respiration in pieces following section leave no doubt that a similar relation between susceptibility and respiratory rate exists in *P. lata*. If this is true, the inference is justified that the reconstitu-tional differences at different levels are in some way associated with the differences in rate of metabolic reactions, as indicated by rate of respiration.

The relations of reconstitutional processes to length of piece also appear to be non-specific and quantitative in character, for they, too, can be altered by the quantitative action of external factors. And, finally, the relation of head frequency to physiological age is apparently non-specific and quantitative and can be altered ex-perimentally by changes in condition which affect primarily rate, rather than kind of metabolic reactions.

Nowhere do we find any evidence for the existence of specific formative substances. Given the specific protoplasm of a plana-rian species, the differences in the reconstitutional processes and results are apparently dependent primarily upon quantitative dy-namic differences rather than upon specific qualitative factors.

Physiological Analysis of Head Frequency.—It has been shown that the head forms differing from the normal which appear in the reconstitution of *P. dorotocephala* represent various degrees of differential inhibition of head development, and that they can all be produced by chemical and physical agents, as well as by physi-ological factors (Child, '16, '20a, '21; Behre, '18; Buchanan, '22). All the experimental evidence supports the conclusion that two antagonistic factors are concerned in the reconstitution of a head— the one positive or determining, the other negative or inhibitory—, and that head frequency in any particular case depends on the relation between these two factors (Child, '14a, '14d, '16, '20a, '21;

Behre, '18; Buchanan, '22). The evidence indicates further that the positive determining factor is the rate of activity of the cells at or near the cut surface (Fig. 31, *x*) which react to section by
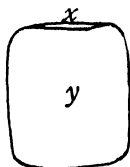
FIG. 31. Diagrammatic outline of piece after section: *x*, region directly concerned in formation of head; *y*, region of correlative inhibitory effect on head development.

dedifferentiation, division, and growth, and are directly concerned in head formation. The inhibiting factor, on the other hand, is apparently the correlative influence on *x* of other parts of the piece (Fig. 31, *y*) which tends to retard or inhibit the dedifferentiation and growth of the *x* cells. More or less excitation of the region *y* occurs temporarily after section, probably largely because of the injury to the nerve cords, and experiments have shown, first, that head frequency decreases as the degree of this excitation increases—*e.g.*, at the more posterior levels of the first zoöid and in shorter pieces—and, second, that inhibition of this excitation increases head frequency. It has been shown further that the differential susceptibility of the regions *x* and *y* and the different degrees of excitation of *y* at different levels of the body provide a physiological basis for altering head frequency experimentally in either direction with the same concentration of a single chemical agent (Child, '16) and with many different agents and conditions (Child, '20*a*; Behre, '18; Buchanan, '22). In short, the facts indicate that head frequency varies directly with rate of metabolism in *x* and inversely with rate in *y*. This relation has been stated in the following brief form:

$$\text{head frequency} = \frac{\text{rate } x}{\text{rate } y}.$$

This formula is perhaps not complete, but serves provisionally to indicate the opposite relation of the two factors to head frequency. If it indicates the relation correctly, it follows that head formation in reconstitution really takes place in spite of the rest of the piece.

In other words, in so far as the x cells become independent of y, they dedifferentiate and begin the development of a new individual, the head arising first as in embryonic development. The various differentially inhibited head forms result from different degrees of inhibition of x by y or by some external factor.

All the experimental evidence at hand indicates that this interpretation holds for *P. lata* as well as for *P. dorotocephala,* the chief difference being that in *P. lata* the inhibiting action of y shows less increase with decrease in length of piece and at more posterior levels of the first zoöid, and that head frequency is therefore normally higher in the shorter pieces and at more posterior levels of the first zoöid and is less increased by inhibition of y in *P. lata* than in *P. dorotocephala.* This difference between the species is what we should expect if the inhibiting action of y on head formation is nervous in character, as the facts lead us to believe. The differences in excito-motor behavior and in development of sense organs certainly indicate a lower degree of nervous organization in *P. lata* than in *P. dorotocephala.* Moreover, the differences in degree of excitation of y following section and in head frequency in relation to level of body and length of piece are less in *P. lata* than in *P. dorotocephala,* and this again suggests a lesser degree of specialization of different body levels in relation to the axial gradient.

The fact that in *P. lata* head frequency is almost as high in young as in old animals, while in *P. dorotocephala* it is much lower in young than in old, also indicates that the region y is less effective in inhibiting head formation at x in *P. lata.* Rate y is higher in relation to rate x in young than in old animals because the tissues of young animals have in general a higher rate of metabolism than those of old, but this difference has much less effect on head frequency in *P. lata* than in *P. dorotocephala.* The more rapid growth reaction in *P. lata* also indicates that y is less effective in this species in inhibiting dedifferentiation and growth of x. The physiological analysis of reconstitution leads, in fact, to the same conclusion as observations on the behavior of the two species, viz., that *P. lata* is a more primitive, a less highly specialized form than *P. dorotocephala.*

Tailless Forms and Biaxial Heads.—Tailless forms develop

from very short pieces in which a single polarity, in these experiments the original polarity, is maintained. They arise when the cells at the posterior cut surface are not sufficiently active in relation to parts anterior to them to grow at the expense of the latter. Heads cut off immediately behind the eyes always remain tailless, and in general when the piece is so short that the posterior cut surface is very close to the new head the development of a new posterior end is inhibited, because the rate of metabolism at other levels of the piece is so high that the cells at the posterior end can obtain but little nutrition.

Biaxial forms arise in very short pieces of *Planaria* when a new physiological gradient arises in relation to the posterior cut surface. In the short piece conditions are most favorable for the origin of such new gradients because there is but little physiological difference between the two cut ends—*i.e.*, these short pieces are nearly apolar because they are short, consequently each cut surface may become a dominant region and determine a polarity in the opposite direction to the other (Child, '15*b*, pp. 98–100). The higher frequency of biaxial heads under ordinary conditions in *P. lata* than in *P. dorotocephala* suggests that polarity—*i.e.*, the longitudinal axial physiological gradient—is less stable in the former, and this also is in accord with the conclusion that *P. lata* is a less specialized form than *P. dorotocephala*.

SUMMARY.

1. *Planaria lata* possesses a short posterior zoöid and undergoes fission. Fission does not interfere with sexual maturity, probably because the level of fission is far posterior to the genital pore.

2. Susceptibility decreases from the anterior to the posterior end of the first zoöid and increases again with approach to the posterior zoöid.

3. Head frequency varies in relation to level of body in the same way as susceptibility. The differences in head frequency at different levels increase as length of piece decreases, and head frequency is slightly lower in young than in old animals.

4. Isolation of pieces is followed immediately by a great increase in susceptibility, CO_2 production, and oxygen consumption, then a gradual decrease occurs during 12–24 hours to a level still far

above that of whole animals. Later a gradual increase coincides with the progress of reconstitution.

5. The increase in susceptibility after section in relation to body level and length of piece varies inversely as the susceptibility gradient of whole animals and the head frequency in reconstitution.

6. The factors determining whether a piece shall or shall not develop a head become to some extent effective within a few hours after section.

7. The experimental data all support the conclusion that in *P. lata*, as in *P. dorotocephala*, two factors are concerned in determining head frequency: one the rate of metabolism in the cells at the cut surface, being positive or determining; the other the rate of metabolism in other regions of the piece, being negative or inhibitory. Head frequency in any particular case is determined by the relation between these two factors. Apparently the inhibitory factor is less effective in the shorter pieces and at more posterior levels in *P. lata* than in *P. dorotocephala*.

8. The physiological analysis of reconstitution agrees with observations on behavior in indicating that *P. lata* is a less highly specialized form than *P. dorotocephala*.

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BIOLOGICAL BULLETIN

MOTOR REACTIONS OF THE FRESH-WATER SPONGE, *EPHYDATIA FLUVIATILIS.*[1]

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Parker's work on *Stylotella heliophila* ('10) has suggested the possibilities of similar experiments with fresh-water sponges and a comparison of the reactions of the two types.

Aside from Parker's monograph practically nothing has been published on the motor reactions of sponges. Dr. R. E. Grant (1825–'26) published a series of papers on the "Structure and Functions of Sponges," which are of historical interest only. He quotes an earlier paper (Ellis and Knight, *Transactions of the Royal Society of London*, 1765), in which these men stated that they had seen the orifices on the surfaces of sponges contract and dilate themselves. Grant studied a large number of salt-water species, both in their natural habitat and in the laboratory. He was the first to notice and accurately describe the currents of water coming from the fecal orifices (oscula) and spoke of them as mysterious currents because he could not learn what caused them. He irritated the orifices and other tissues with corrosive acids, red hot wires, etc., but under no circumstances did he see the osculum closed and erroneously concluded that there was a complete lack of irritability and contractility, attributing the statements of Ellis and Knight concerning the "systole and diastole of the fecal orifices" to "some optical deception a little assisted by the imagination."

Parker made two types of experiments on *Stylotella*, noting

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the responses of the oscula, ostia, and choanocytes first to mechanical and then to chemical stimuli. He studied the reactions of the sponge to flowing water, injuries, and cuts. Likewise he studied the effects which ether, chloroform, cocaine, strychnine, and atropine produced upon the sponge and compared his results with the known effects of these drugs on smooth muscle and on ciliated tissue.

Ephydatia fluviatilis (L.), the fresh-water sponge with which the experiments described in this paper were made, differs so greatly in structure from *Stylotella* that it was impossible to duplicate many of Parker's experiments. The responses of the oscula were watched to determine the effects of various mechanical stimuli such as injuries, electrical stimulation, and changes in temperature. The purpose was to determine the effects ·of such stimuli and the rate of possible transmission of these effects from one part of the body to another.

All the sponges used in the following experiments were collected in small ponds along the railroad tracks in the neighborhood of Hammond and Buffington, Indiana. They were carried to the laboratory in fruit jars and then transferred to large jars of slowly running water.

The ponds from which the material was collected [1] were shallow, often almost filled with bulrushes. The bottoms were covered with mud and cinders. The sponges were, for the most part, growing on the under side of old railroad ties floating in the water and were more or less cushion-like in form and without much branching. Some, however, were found growing on the submerged parts of plants or on leaves, but here their form was more spreading, following that of the body to which they were attached.

The body of the sponge is dotted all over with very small dermal pores or ostia. The oscula are relatively prominent little chimneys of dermal membrane, standing our ·from almost any point on the suface. Bundles of smooth, pointed, and almost straight silicious spicules make up the skeleton. The internal canals of the body meander irregularily between these bundles of spicules. The dermal membrane, which completely envelops

[1] See Shelford, "Animal Communities in Temperate America," Chapter VIII.

the body, is held up in tent-like elevations by the protruding ends of the spicules.

The oscular chimneys seem to be kept open by the pressure of the currents of water passing out through them. They are continuously changing their form. At times one will be long and slender; again it will be shorter and much thicker or even dome-like, the base being as much as three or four times the height. In instances of the first type the mouth of the chimney will probably be open almost to the width of the diameter, although these mouths are frequently seen to be nearly closed. In the type last mentioned they are usually small.
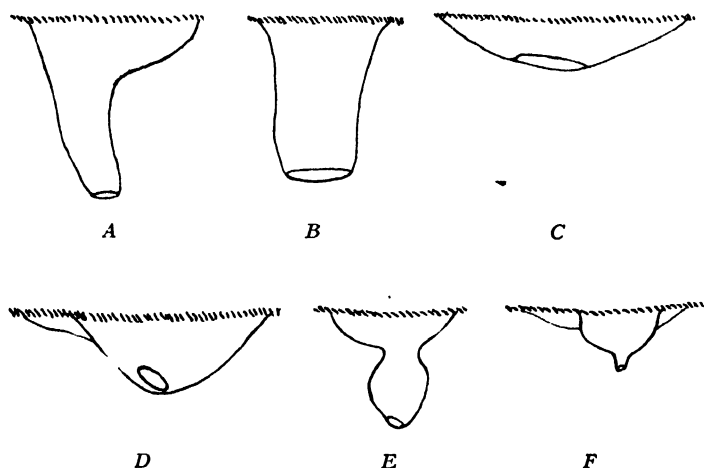
FIG. 1.   Various forms in which the same oscular chimney was observed.

These variations in shape lead to the belief that the entire chimney contains sphincter-like bands. If all such bands were about equally contracted the result would be a long slender chimney, Fig. 1, A and B. If only those at the end contracted and the others relaxed, the result would be the dome-like shape, Fig. 1, C and D. Several instances of a contraction of the terminal sphincter and also of the chimney near its base, with a relaxation between, were noticed. The result was a sort of globe-like chimney attached to the sponge body by a narrow neck, Fig. 1, E. The chimneys also seem capable of a decided shortening without much change in diameter. This may be

brought about by a contraction of longitudinal fibers, but it could not be definitely demonstrated.[1]

EFFECTS OF CURRENTS OF WATER ON THE OSCULUM.

Potts ('87) makes the statement that he found *E. fluviatilis* in both quiet and running water. It seems to grow best in ponds of quiet water, but very fine specimens are found on the under side of rocks in rapids or at the base of water falls where the river current is the swiftest.

To get an idea of the effects of water currents, a few sponges were placed in a flat dish containing about a quart of water and left over night. The next morning the oscula were still expanded, and, by watching the movements of tiny particles of suspended material in the water, steady currents could be seen flowing through them. This test was repeated with the same results. It was noticed, however, that if the sponges were left in such a dish of unchanged water much longer than a day they gradually became less active and in many cases soon died.

To determine the effect of a swift current, a glass tube was connected with the water tank and placed in one side of a dish containing the sponges. The water was turned on at full force and left running for about four hours. After transferring the sponges to a watch glass for observation (without taking them from the water) it was found that the entire chimney had contracted until it was almost flat, but that the mouth of the osculum was wide open and currents of water were coming out. The following illustration shows the extent of the contraction of the chimney.

FIG. 2. A chimney before and after a strong current of water had been applied, showing the extent of the contraction.

[1] See, E. A. Minchin, Lankester's "Treatise on Zoölogy," p. 44.

Within thirty minutes after taking the sponges out of the swift current the chimney had again extended to about half of its original length. The osculum was slightly contracted.

The current had caused the sponges to roll and tumble over the bottom of the dish to some extent. To find out if this had been the cause of the shortening of the chimneys, the sponges were placed in watch glasses which, in turn, were placed within the larger dish. When the water was turned on, the force of the current against the curvature of the watch glass held the sponges in place. This time a swift current flowed over the sponges but they did not roll about. Within twenty minutes the chimneys had flattened out as before.

The sponges were now placed in a large jar in which a slow current of water was kept going. After being there for a few hours the chimneys were found to be well extended and with strong currents coming out through them.

Neither the absence of currents nor the presence of strong currents causes the osculum to close although the latter does cause a general shortening of the oscular chimney. In small quantities of water, however, the sponges soon became less active and in time died; the smaller the quantity of water the sooner the changes could be noticed. This effect could not have been due to the absence of the mechanical stimulus of flowing water, because if placed in a large tank of quiet water the sponges will live for a long time. Nor could it have been caused by lack of oxygen, because the dishes which were used were large and shallow so that the water was well exposed to the air. It must have been produced then by self-poisoning from the products of metabolism that accumulated in the small quantity of water. The greater general vitality of the sponges when placed in slow running water or in large tanks of quiet water is accounted for by the fact that these products were removed as they were thrown off by the sponges or were diffused through the large volume of water.

These results are entirely different from those obtained by Parker from somewhat similar tests with *Stylotella*. When he transferred *Stylotella* from the natural habitat to tanks of quiet sea water, the oscula invariably closed within ten minutes and

did not reopen until they were placed in running water. He was able to prove that the mechanical stimulus of the currents of water on the outlet tip of the finger where the osculum was located, caused it to open. The oscular closure seems to be a protective adaptation of *Stylotella*, which is a shallow salt-water species frequently left exposed to the air when the tide is out. Since the fresh-water sponge is never exposed to such tidal conditions, one would not expect to get the same reactions.

BRUSHING.

Rubbing the sides of the chimney with a needle, prodding it gently, or even inserting the needle into the mouth of the osculum and rubbing the inside of the chimney, caused no noticeable reaction; however, if the needle were carefully rubbed around the edge of the mouth of the osculum, an immediate contraction of the orifice would follow and it would continue to contract for about three minutes. Following the contraction of the sphincter at the orifice a wave of contraction would travel down the chimney although the amount of contraction would be less than at the orifice. On a chimney of 1.5 mm. length it would require about six seconds for this wave to run from the tip to the base of the osculum. In the next three or four minutes it would again expand to its original size. The edge of the mouth of the osculum, therefore, seems to be more sensitive than the rest of the chimney. Concerning the reactions of *Stylotella*, Parker states that stroking an open osculum with a bristle or brush does not cause it to close, but if it is closed, this treatment may at rare intervals induce it to open.

SHARP BLOW.

Several times while getting the electrodes into position to work with the inductorium one of the wires of the electrode would catch on a spicule and, springing loose, strike an oscular chimney. Each time the entire chimney would shrivel up immediately in a more or less collapsed condition, but within twenty or thirty minutes it would be open and functioning as before.

CUTTING.

Cutting into the body seemed to have no effect in the way of induced movements. With a small scalpel an incision was made

into the body of the sponge 3 mm. from the base of the chimney without causing any change in the rate of the currents or in the size of the chimney. Another cut right at the base of the chimney caused it to close. It did not reopen for two days. This cut was so near the chimney, however, that the closing was probably due to cutting into the chimney cavity and when this had healed over the chimney again expanded. The result of such injuries differs from the effect of similar ones on *Stylotella*, where a cut from 3 mm. to 5 mm. from the osculum was found to cause closure within nine minutes.

PIN STICKING.

Sticking a needle into the flesh of the sponge at a distance of 2 mm. from the osculum caused no noticeable effect on the osculum. The same type of injury 5 mm. from the osculum of *Stylotella* resulted in the osculum closing within ten minutes and remaining closed for several hours.

EXPOSURE TO THE AIR.

A sponge was lifted from the water and held in the air for three minutes. A large chimney was noticeable before, but could not be found after placing the sponge back in the water; neither could it be found at any later time.

THERMAL RESPONSES.

The average temperature of the water in the tanks in which the sponges were kept was about 27° C. Eight of the sponges which had well developed oscular chimneys and from which strong currents of water were flowing were placed in dishes in a refrigerator which had a uniform temperature of 5° C. and left until the water had been cooled to 7° C. They were examined at regular intervals. At 16° there was no noticeable change in any of the sponges. At 11° all had a shriveled appearance, some of the oscula were open slightly, and very slow currents coming from them were barely visible. At 10° no oscula could be found, and at 7° the dermal membrane had completely shrunk in over the body of all the sponges and no sign of activity could be detected. The dishes were transferred to an incubator and

observations were made until the temperature of the water had reached 40° C. At 24° the dermal membranes had taken on their usual appearance, the oscula were somewhat expanded and currents were issuing from them. At 34° the chimneys were more extended and stronger currents were coming from them than at any previous time. At 37° they were still expanded but were slightly flabby and the currents were weaker. At 40° the oscula had all disappeared, the dermal membranes were completely shriveled. None of the sponges recovered after being raised to the temperature of 40°.

Comparing these results with those obtained by Parker, it will be seen that the salt-water sponge is apparently less affected by temperature conditions than is the fresh-water species. With *Stylotella* at between 9° and 10° the oscula and ostia remained open and the only effect seemed to be that the currents became slow. At 40° there was a slight constriction of the osculum and the currents gradually became slower and stopped. At 45° there was a flabby contraction of the oscula and the currents ceased abruptly. They did not recover after being raised to the temperature of 45°.

LIGHT.

Neither full sunlight nor complete darkness seemed to have any effect on *E. fluviatilis*. One large, healthy specimen was placed in a dark cupboard and left there four days. At the end of this time the green color due to contained algæ had faded, but the activity of the sponge in producing currents had not diminished in any way.

ELECTRICAL STIMULATION.

By using an inductorium with electrodes of fine platinum wires, the effect of weak Faradic stimulation could be observed. The electrodes were applied to three different places: first, on the side of the chimney; second, at the base of the chimney, one wire on each side; third, at points on the flesh immediately back of the chimney at distances of from 3 mm. to 5 mm. from it. A weak stimulus when applied to the tip of the chimney caused a contraction of the oscular sphincter and a gradual

wave of contraction down the entire length of the chimney. This wave traveled down at the rate of about 1.5 mm. in four seconds. A strong stimulus applied to the chimney caused a complete relaxation, as though paralyzed, and from which it did not recover.

STIMULATION AT THE SIDES OF THE CHIMNEY.

Choosing chimneys which were long and well extended the electrodes were applied at one side of the chimney, Fig. 3, *A*. When a weak stimulus was used, the response required about one minute before it appeared. A mild stimulus resulted in an immediate response. First, the chimney would bend toward the electrodes, the bending occurring at the points where the wires touched the chimney, Fig. 3, *B*. This bending always occurred within thirty seconds from the time the stimulus was started. At the same time, but continuing longer, the entire chimney would shrink slightly. This slow shrinking continued for from one to five minutes. If the wires were applied to the opposite side (Fig. 3, *C*) while the chimney was still bent as in (*B*), within one minute the end of the chimney would swing over in that direction, Fig. 3, *D*. Within thirty minutes the chimneys would straighten and expand to their former size and shape.
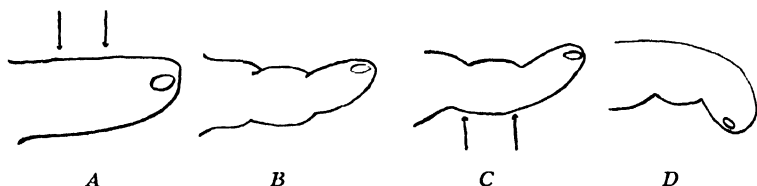
FIG. 3. The arrows indicate the points on the side of the chimney where the electrodes were applied.

STIMULATION AT BASE OF OSCULUM.

When a very weak stimulus was given for fifteen seconds with an electrode at each side of the base of the chimney, a very gradual longitudinal shrinking could be observed. The time required for the contraction to travel from the base to the tip was ten seconds, a rate of transmission of 0.17 mm. per second.

If the stimulus was a little stronger, an immediate contraction was followed by a slower general contraction, both lengthwise and in circumference, which would continue for about fifteen minutes, Fig. 4, *A* and *B*. The chimney would then begin to expand and within thirty-five minutes would be almost as large as before. On one occasion the sphincter at the base contracted more quickly than the others, resulting in the form of chimney shown in Fig. 4, *C*.

A   *B*   *C*
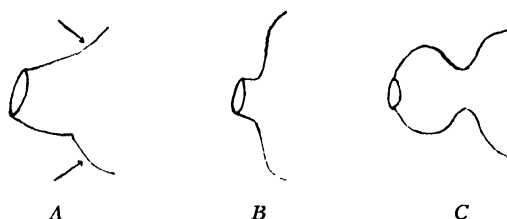
FIG. 4. The arrows indicate the points on each side of the base of the chimney where the electrodes were applied.

STIMULATION ON THE FLESH.

On several sponges the electrodes were applied immediately behind the osculum at distances of from 4 mm. to 2 mm. from it, and the flesh stimulated for periods varying from fifteen to sixty seconds. No effects could be seen on the chimneys.

There was, however, some indication of an excitation of the choanocytes. The currents were stronger and more rapid for from ten to fifteen minutes after the stimulation. Although this was taken as an indication of greater activity on the part of the flagellate cells, it is possible that the stimulation caused the ostia to dilate or open wider and in this way allow a larger volume of water to pass into the sponge body. It does not seem, however, that this would account for the increased rapidity with which the currents poured out of the oscula.

There was a noticeable difference in the responses of the oscula to Faradic stimulation depending on the strength of the electrical current used. From a weak stimulus,[1] a very gradual contraction was followed by a more rapid relaxation, while, if

[1] By a weak stimulus is meant one which could barely be detected when the electrodes were applied to the tongue, while by a mild stimulus is meant one that could be felt when the electrodes were applied to the lips.

the stimulus was mild, there would be an immediate contraction followed by a slower more general contraction.   In this case the chimney would remain contracted for a few minutes and then expand much more slowly than it had contracted.   In the second instance, all of the contractile cells probably were directly stimulated by the electrical current, while in the first case it is probable that only those where the electrodes were applied were directly stimulated, the gradual contraction being the result of transmission of the stimulus from one cell to the next.

In one instance, following a very weak stimulation for thirty seconds, it was possible to keep an accurate measurement of the time required for contraction and relaxation of the sphincter at the mouth of the osculum.   No check was made on the general contraction or relaxation of the chimney.   That the contraction was slower than the relaxation is shown by the following graph. The temperature of the water was about 27° C.
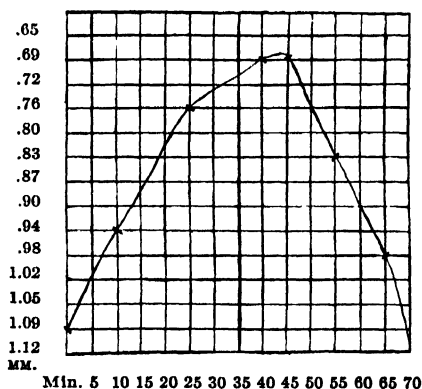
FIG. 5.   Graph showing the rate of contraction and of relaxation of the sphincter at the mouth of an osculum following a weak Faradic stimulation.   The abscissa represents the intervals of time in minutes and the ordinate the diameters of the opening in millimeters.

TRANSMISSION OF STIMULI.

It has already been pointed out that, apparently, very little transmission from one part of the body to another resulted from the application of the various types of stimuli used in these experiments.   To determine if there was any transmission at all,

a sponge which had two large oscula at a distance of about 5 mm. apart was selected. A strong stimulus was applied with the electrodes at the base of one chimney. The result was an immediate jerk of the entire chimney followed by a gradual and complete relaxation of the entire chimney from which it did not recover. The other chimney was not affected.

There is, however, a noticeable transmission of stimuli in the chimney itself and the rate of this transmission is more rapid from the tip to the base than from the base to the tip. This is shown in the experiments with electrical stimulation and by rubbing the chimney with a needle. Rubbing the edge of the mouth of the osculum with a needle started a downward wave of contraction which could be seen to travel at the rate of about 0.25 mm. per second. Rubbing at the base produced no effect. Weak Faradic stimulation at the tip caused a downward wave of contraction with a rate of about 0.35 mm. per second, while stimulation at the base produced a slight wave of contraction which traveled toward the tip at the rate of about 0.17 mm. per second.

CONCLUSIONS.

Ephydatia fluviatilis is a fresh-water sponge which lives in either quiet or flowing water. Swift currents cause a shortening of the oscular chimney but do not cause a constriction of the osculum. Placing the sponge in a small quantity of quiet water results in a cessation of activities and death of the sponge in a short time, probably because of the metabolic products which accumulate in the water and poison the sponge.

The edge of the mouth of the osculum seems to be more sensitive to stimulation that the remainder of the chimney, as shown by the effect of rubbing it in various places with a needle.

Cutting the body of the sponge or sticking a needle into it seemed to have no effect except the local effect on the tissues which were injured. That there is no transmission of the effects to surrounding tissues, even for a distance of 2 mm. or 3 mm. from the injury, is shown by the continued functioning of the parts as indicated by the currents coming from the oscula and

by the fact that the chimney did not contract or in any way change its shape.

Subjecting the sponge to a low temperature does not have as disastrous an effect as increasing the temperature. Between 20° amd 30° C. seems to be approximately the temperature at which it thrives best. It is unable to live at a temperature of 40° C.

The oscular chimneys being continuous with the dermal membranes and themselves being contractile, one would expect to find the dermal membrane also contractile. It is capable of contraction to a slight extent under certain conditions. The chief contractile fibers, however, seem to be found only in the sphincters of the chimneys, which probably work against the general pressure of the water going out through the chimney.

Although in most cases, the responses of the fresh-water sponge were similar to the responses of *Stylotella*, there is one noticeable difference. This sponge responds to stimuli much more slowly, and the rate of transmission, where any at all is observable, is much slower.

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STUDIES IN MARINE ECOLOGY:
I. THE DISTRIBUTION OF COMMON LITTORAL INVERTEBRATES OF THE WOODS HOLE REGION.

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The distribution of animals within the Woods Hole region has been well studied by men interested in individual species and by those concerned with general faunistic problems. Some aspects of the ecology of the region are thoroughly set forth by Verrill and Smith in their "Report on the Invertebrate Animals of Vineyard Sound" made fifty years ago. This classic study remains the best account of the ecology of littoral species available.

The extensive "Biological Survey of the Waters of Woods Hole and Vicinity" [1] completed about ten years ago by Sumner, Osburn and Cole, while a mine of information concerning the animals of the region, was directly concerned with dredging operations and has little to say at first hand concerning the animals of the intertidal region or those found just below the tidal zone. Among other suggestions they recommend (p. 25) that the intertidal fauna should receive the same detailed attention that they have given to the bottom dwelling species.

In the absence of a report by the person best qualified to write on the subject, Mr. George M. Gray, the present series of papers has been prepared to make available information accumulated in nine consecutive summers' experience with the inshore invertebrates of the region.

The work has been done in connection with a teaching appointment in the course of Invertebrate Zoölogy of the Marine Biological Laboratory. It represents the collaboration of eighteen staff members and of about four hundred students. Many of the present collecting methods were installed in conjunction with Professor Caswell Grave, my predecessor in charge of the

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[1] This report will be referred to hereafter as the *Biological Survey*.

course, but the records here used have been kept from the beginning by myself with the occasional help of other staff members.[1]

The records are based on the bi-weekly collecting trips of the Invertebrate Class and cover most thoroughly the period from about June 20 to August 15. These trips have been supplemented by expeditions made by instructors and by special trips for particular observations.

The organization of field work for eight years has been to divide the class into as many collecting teams as there were instructors. One person from each team was appointed recorder for the day and was supplied with a list of all the animals previously taken from the locality under consideration. The animals found were recorded according to habitats. The complete list for the year was made up from these combined records.[2]

[1] The following people have been at one time or another members of the instructing staff of the Invertebrate Course and have contributed to the data on which this series of papers is based. Without their coöperation this work could not have been done. Caswell Grave, Raymond Binford, E. J. Lund, George A. Baitsell, T. S. Painter, F. M. Root, W. J. Kostir, Robert H. Bowen, C. L. Parmenter, G. S. Dodds, Robert Chambers, Jr., Ann H. Morgan, W. J. Crozier, Donnell B. Young, J. P. Visscher, J. A. Dawson, Christianna Smith and E. A. Adolph.

I am indebted also to Mr. G. M. Gray for much valuable aid and friendly assistance; to Dr. Mary J. Rathbun for identification of the Brachyura; to Mr. Waldo L. Schmidt for similar service with the Anomura and Macrura; to Mr. Clarence R. Shoemaker for similar service with the amphipods and isopods; to Professor E. S. Morse for assistance with some of the molluscs and to Professor Raymond Osburn for assistance with the Bryozoa.

[2] The formal record of collecting experience has been recorded in abbreviated form on library cards which are deposited in the Library of the Marine Biological Laboratory. An annotated catalog of the distribution has been prepared as Study II. of this series and deposited with the library of the U. S. Fish Commission who have kindly agreed to furnish copies to the libraries of the Marine Biological Laboratory at Woods Hole; the Museum of Comparative Zoölogy at Cambridge, Scripps Institution at LaJolla; the United States National Museum at Washington and to the Harpswell Laboratory at Mount Desert Island, Maine.

The catalog shows the littoral invertebrates collected during the years 1915–1921 inclusive. Each locality in which an animal has been taken is recorded. The number of years which it has been found in a given locality is shown and an index figure of comparative abundance is also given. Where possible and desirable the location of particularly favorable collecting grounds is given with some exactness. This elaborated catalog forms the basis from which the facts presented here are drawn and together with the present report gives the background for the two following studies.

So far as possible, identification was done in the field. Doubtful specimens were referred from one instructor to another. Specimens new to the locality or difficult to identify were brought into the laboratory for further study. With the exception of the arthropods, few of the specimens have been referred to experts although we have gradually accumulated a type collection of animals found. The identification of animals has been made on the conservative basis that when doubt existed, the specimen was referred to the more common species. Wild identifications have been eliminated as far as possible, even to the extent of throwing out the entire reports of inexperienced instructors.

In spite of this care, mistaken identifications have probably been turned in and accepted. The list here given is substantially correct since the animals have either been reported by qualified collectors or placed on the list from demonstrated specimens· The imperfections lie largely in failing to distinguish closely related species and in possible errors in distribution records.

II.

The collections upon which this series of reports are based have been made largely in the littoral zone as defined by Edward Forbes; that is, between high water and a depth of two fathoms. This is not the littoral zone of modern zoölogists, but the term has been used with so great a variety of meanings that the extent of the study can be more easily and definitely located as being in the intertidal and ad- or sub-tidal regions.[1]

The intertidal zone is much restricted in the Woods Hole region on account of the slight· rise and fall of the tides. The

[1] Murray and Hjort use the term "littoral zone" to include the region near the shore down to a depth of 36 or 40 meters: "almost as far as there are sea-weeds." It is frequently used as by Petersen to include the entire continental shelf. The botanists tend to be more exact. Kjellman limits the term to the region between extreme low and extreme high tide. Davis regards the littoral zone as extending from about mean low water to the highest point at which algæ can grow. Flattely and Walton ('22) follow Cotton ('12) and define the littoral region as extending from the level of highest marine vegetation, to low water at neap tide.

I prefer to use littoral in its original meaning of "pertaining to the shore"; intertidal or tidal zone adequately and exactly describes the region between the tide lines, and sub-tidal or adtidal are exact terms, if not the most correct etymologically, for the region below low tide. The question is discussed in the *Biological Survey*, p. 179.

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tide tables of the U. S. Bureau of Commerce show a spring tide range of about five feet for this section of Buzzards Bay and only about two feet for Vineyard Sound stations.

Studies have been made in the following localities:[1]

WHARF PILINGS.

Crane's Wharf Pilings.—This is a comparatively new wharf located near the public steamboat wharf in Great Harbor at Woods Hole. At the shore end the water at low tide comes close to the retaining wall and some collecting has been done annually in the crevices of the wall. The water at the outer end is over twelve feet deep. The number of species and of animals on these pilings has increased noticeably during the period of observation.

Vineyard Haven Wharf Pilings.—The old New York and Portland Wharf on the south side of Vineyard Haven is located well out toward the Sound. The water here comes up on a sandy beach which at low tide is bare for a considerable distance under the wharf. At the outer end the Government Chart shows 11 feet of water. In my experience the water is deeper. This is an old wharf with many pilings rotted off below water level. Some of the pilings are reproduced in the American Museum of Natural History in New York. Collections from both wharfs were made from boats by means of the usual scrape nets.

Marine Biological Laboratory Pier on Glass Slides.—For a number of years, glass slides have been placed under the M. B. L. pier in connection with other studies. In 1921 the slides were carefully examined by Dr. D. B. Young and myself after they had been suspended in water under the pier from July 1 to August 9 at a depth of about six feet. The M. B. L. supply float containing animals from all parts of the region was only a few feet away and accordingly more species were attached than might normally be found.

ROCKS AND FLATS.

Hadley Harbor, Southwest and Southeast Gutters.—These naturally narrow rocky gutters have been further narrowed artificially

[1] Consult map.

MAP
SHOWING PRINCIPAL
LOCAL GEOGRAPHICAL FEATURES
REFERRED TO IN ACCOMPANYING REPORT
BASED ON U.S.C.&G.S.
CHART NO. 112
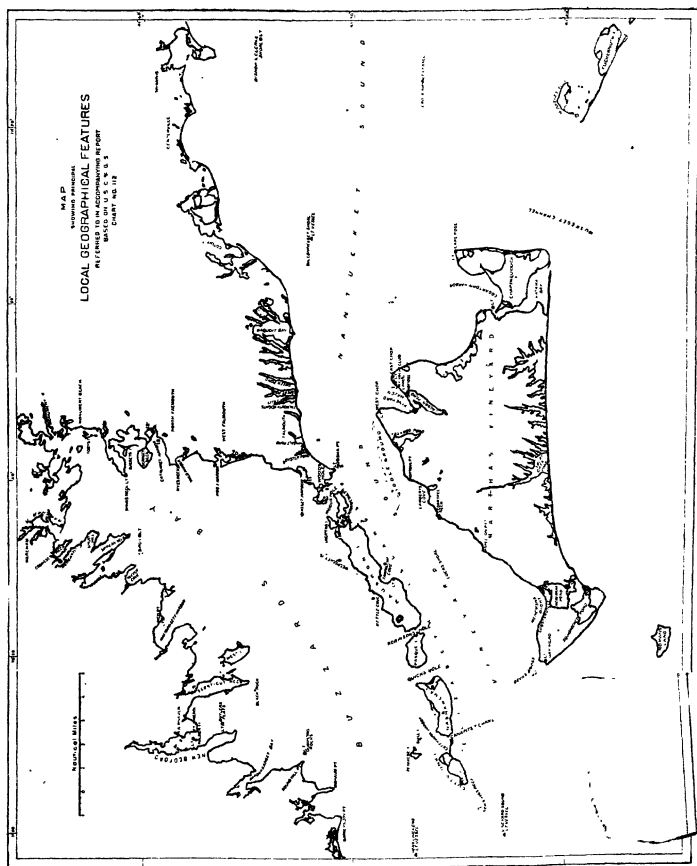
1. Vineyard Haven Wharf.
2. Station 1, Vineyard Sound; near oyster beds.
3. Station 2, Vineyard Sound; *Chaetopleura* grounds.
4. Station 3, Vineyard Sound; *Amaroecium pellucidum* grounds. Locality 1 of the dredging in Great Harbor is just within the harbor entrance.
5. Crane's Wharf. Localities 2 and 3 in Harbor dredging are nearby.
6. Lackey's Bay and Sound Gutters. The figure stands approximately on Blind Gutter Bar.
7. Station 4, Vineyard Sound; starfish hole.
8. Station 5, Vineyard Sound; sand dollar bed.
9. Kettle Cove.
10. Northwest Gutter Flats.
11. Ganett Bay.
12. North Falmouth Flats.
13. Squeteague Harbor in the North Falmouth Flats complex. The map is taken from Bull. 31, U. S. Bureau of Fisheries.

so that they can be bridged. Strong tidal currents run through them the greater part of the day. They are relatively shallow, rock-walled channels, containing about six feet of water and are connected with open water by creeks which are also rock edged. Small patches of mud and sand occur frequently and the whole system of protected waterways supports many plants, *Ascophyllum*, *Fucus* and *Sargassum filipendula*.

Hadley Harbor Flats, Northwest Gutter.—Northwest Gutter separates Uncatena Island from Naushon. Before opening into Buzzards Bay it enlarges to form an approximately square expanse of shallow water about 250 yards along the south and west sides and about 400 yards in greatest diagonal. In most places the water is so shallow that it is difficult to push a boat along at low water. The sand bar over the channel of the gutter is fully bare at extreme low tide often to the extent of an acre or more.

The wide channel is kept scoured clean by the current, but behind the protecting sand and gravel spits, organic debris has accumulated to the depth of several feet and supports a rank plant growth composed chiefly of eel grass. At the Bay entrance there is the usual accumulation of rocks which extend off to a sand bottom some four feet below the lowest tide. The mud flats are bordered by rocks partially buried by mud.

Gansett.—Gansett is an offshoot of Quamquissett Harbor and has the same opening into Buzzards Bay. The main axis extends at an angle from the opening so that the back portion is usually protected from the direct drive of the waves. The opening is about 200 yards wide and the bay is approximately twice that length. At mid-mouth at mean low tide the water is 18 feet deep. The sides slope in rapidly near the shore so that there are only narrow strips of the different habitat zones. At the sides are the customary rocks and the outer corners are guarded by rock piles. At the head of the bay the shore is sand mixed first with gravel and lower with mud. Eel grass comes within two rods of the water's edge at low tide and thickly covers the bottom throughout its extent.

North Falmouth.—The collecting grounds here are scattered. They are located at the head of Cataumet Harbor and extend

over Squeteague Harbor which opens from the former by a winding narrow passage. Except for the dredged passage, most of the region can be waded at low tide. Much of the ground in Cataumet and almost all in Squeteague Harbor is left bare by the spring tides. The collecting is over a wide range of bottom: sand, mud, scattered rocks and gravel with and without eel grass and other sea-weeds. There is a somewhat sparse collection of rocks along the shore line.

Lackey's Bay.—Lackey's Bay belongs to the Hadley Harbor complex. It is located on the Vineyard Sound side between Naushon and Nonamasset. The part studied forms an expanded entrance to Middle Gutter which, by the construction of a causeway, has become Blind Gutter. The current is much diminished by the causeway and the inner part of the bay is deeply overlaid with muck. Eel grass is abundant. The region most studied is about 400 yards long by 200 yards wide and is separated from the Sound by a sand bar which is left bare at low tide.

DREDGING.

The dredging has been largely in three localities in Vineyard Sound. These are the sand dollar bed (Map, No. 8) near the east side of the entrance to Tarpaulin Cove in about 20–30 feet of water. The bottom material brought up by the coarse dredge used is largely composed of shells. The starfish hole (Map, No. 7) is further east and still off Naushon, has about 90 feet of water. The *Chætopleura* grounds (Map, No. 3) off Nobska have about sixty feet of water. The bottom is decidedly pebbly. Some dredging has been done further east on or near the planted oyster bed (Map, No. 2) in Falmouth Harbor. The bottom here is sand and gravel in about 60 feet of water. In 1921 we dredged off the west entrance from Vineyard Sound to Great Harbor (Map, No. 4) in about 80 feet of water. This is over an *Amarœcium pellucidum* bed.

In 1920 we dredged in Great Harbor (Map, No. 5): at the east end of Nonamasset in 10–12 feet of water; in the Fish Commission Hole at a depth of 50 feet and at the West end of the passage in Woods Hole in about 20 feet of water.

The dredging work has been largely incidental and the results are given chiefly as a means of comparing the more extensive

results obtained by the dredgings of the *Biological Survey* with our main work further inshore.

A Habitat Check List of the Common Invertebrate Animals of the Woods Hole Littoral with Distribution Records for 1920 and 1921.

The appended list of animals is based on all the collecting done since 1912. The distribution records are based on the reports from operations in 1920 and 1921. The statistics given are from team records. Thus in these two years, two teams collected *Chalina* from the mud, eight teams have recorded it from rocks, ten from wharf pilings and nine from dredging. The figures given show no indication of the number of specimens taken other than that suggested by the fact that the more animals present, the greater the probability that all teams would find them. Anyone interested in the abundance of these animals in. particular localities is referred to the second study in the present series.

The tabulation is from the reports of 30 collecting teams operating on wharf pilings; 52 from sand, mud, gravel and eel grass; 56 teams from rocks and 42 from dredging. The records of plankton have been kept in a different way and the presence of recognized animals in late July or early August is merely checked.

The classification of habitats in the field has sometimes been left to the judgment of the student recorder and it is entirely probable that some of the 52 recorders thought a given habitat was best recorded as "sand" while others regarded animals from a similar place as "mud" dwelling. All gradations between the two exist and the conditions under which the collecting was done do not permit a more refined grading. The error arising from this source is somewhat compensated by the fact that no dragnet collections were made as it was desired to find where individual animals live as well as to collect different species.

Unidentified animals have not been included in the habitat list unless the genus, at least, could be determined with some assurance. All the records are for living animals since for ecological purposes the recording of dead shells can only be worthless and confusing in a region where tidal currents run strongly and where the shore birds distribute shells even over the land.

The nomenclature follows Pratt wherever the species are listed in his *Manual*. Other species have the name given them in the catalog of the *Biological Survey*. No attempt has been made to give synonyms since these can usually be found in the *Survey* catalog.

The arrangement of species within the major divisions is alphabetical. While this does violence to all principles of taxonomy, the taxonomic sense is not strongly developed at present, and this method renders the material more easily available to the average zoölogist than would be the case if a strictly taxonomic system were followed.

The records given in the habitat list are necessarily abbreviated. Thus, *Hydractinia* is recorded as taken from "sand" when the whole record should read: "on shells inhabited by hermit crabs, taken on sandy bottom"; or other animals, as *Sagartia luciæ*, recorded from "mud" which does not mean that the anemone was growing on the mud but that it was found attached to a bit of board or rock surrounded by typical mud conditions.

The classification headed "eel grass" includes records of animals living free among the eel grass, as *Pecten;* attached to eel grass, as *Pennaria;* crawling over it, as *Ophioderma;* on the substratum at its base, as *Microcione;* or burrowing in the substratum at its roots, as *Cumingia*. In addition the lumping is still greater for one must remember that eel grass begins to grow on fairly pure sand and extends back to the pure muck of the flats.

A number of animals are recorded under "rocks and rock-weeds" which were taken only from the substratum under or among the rocks. Whenever all the records are for an animal so found, the entry has been appropriately labeled.

Under "range" is listed the information at hand showing the distribution of the animal along the Atlantic Coast. The abbreviations are: N., north ranging; S., south ranging; M., approximately mid-range; L., local; C., cosmopolitan. Whenever an animal is known to extend twice as far north of Woods Hole as south, it is listed as north ranging and *vice versa*. (Cf. Hoyle, 1889). Some relations between the local and geographical distribution will be discussed in a later paper.

Species.	Range.	Unclassified.	Mud.	Sand.	Gravel.	Eel Grass.	Rocks and Rockweed.	Pilings.	Dredging.	Plankton.
No. Teams Reporting.			52	52	52	52	56	30	42	
PROTOZOA. List not given.										
PORIFERA.										
Chalina arbuscula	S.	2					8	10	9	
Cliona celata (?)	S.			2		1	33	25	16	
Grantia ciliata	N.			1			11	30	13	
Leucosolenia botryoides	N.						13	24	1	
Microciona prolifera	S.	1				4	43	30		
Tethya gravida	L.	1							1	
CŒLENTERATA. *Hydrozoa*										
Abietinaria abietina	N.								12	
Bougainvillia carolinensis.	S.					1	2	1		√ sp.?
Campanularia calceolifera								1		
Campanularia sp.?							1	5		
Clava leptostyla	N.						1			
Clytia grayi	L.						2			
Clytia bicophora	N.						1	1	1	√ sp.?
Clytia cylindrica							1			
Eucheilota sp.? *medusæ*										√
Eudendrium album							2	4	6	
Endendrium ramosum	M.						7	22	7	
Eudendrium tenue								1		
Gemmaria gemmosa	S.		1				1	1		√
Gonionemus murbachii	L.									
Hydractinia echinata	N.	9	18	3		5	12		11	
Obelia bicuspidata	S.						1	1		
Obelia commissuralis	S.	1				12	8	11	2	√ sp.?
Obelia geniculata	N.					5	6	7		
Pennaria tiarella	S.					14	4	10	4	√
Phialidium sp.? *medusæ*										√
Podocoryne carnea	N.	2				1				
Schizotricha tenella	S.						1	11	2	
Sertularia pumila	N.					11	30	12	10	√
Stylactis hooperi	S.									
Tubularia crocea	S.							30	7	√
Scyphozoa.										
Aurelia flavidula	M.									
Cyanea capillata arctica	N.									
Dactylometra quinquecirrha	S.									
Haliclystus auricula	N.									

Species.	Range.	Unclassified.	Mud.	Sand.	Gravel.	Eel Grass.	Rocks and Rockweed.	Pilings.	Dredging.	Plankton.
							No. Teams Reporting.			
			52	52	52	52	56	30	42	
Anthozoa.										
Astrangia danæ	S.						11	7		
Alcyonium carneum	N.									
Edwardsia elegans	N.		1	5		1				
Eloactis producta	S.			3						
Metridium dianthus	N.						29	24		
Sagartia leucolena	S.		3	3		2	37	11	4	
Sargartia luciæ	S.		2	2		6	38	6	2	
Sagartia modesta	S.					4	12 under			
PLATYHELMINTHES.										
Turbellaria.										
Bdelloura candida	S.		3	5	2		7			
Bdelloura propinqua		1								
Polychœrus caudatus	N.		1	3		5	2	1	1	
Procerodes wheatlandi	N.						1			
Stylochus ellipticus	N.		3	2	1	1	6	3		
Stylochus zebra	L.						1		1	
Syncœlidium pellucidum						2				
Nemertini.										
Amphiporus ochraceus	S.							5		
Cerebratulus lacteus	S.		7	11		2	2 under			∨
Cephalothrix linearis	N.						1 under			
Lineus sp.?			2	6		5	1		2	
Lineus bicolor	S.		1	1			1	2		
Micrura leidyi	M.		6	15		8	3 under			
Tetrastemma vermiculum	S.							5	3	
NEMATHELMINTHES.										
Pontonema marinum	S.		1	1		5	6	19	6	
ECHINODERMA.										
Asteroidea.										
Asterias forbesi	S.		1	8		4	28	14	29	∨
Asterias vulgaris	N.							1	4	
Henricia sanguinolenta	N.						6	4	11	

Species.	Range.	Unclassified.	Mud.	Sand.	Gravel.	Eel Grass.	Rocks and Rockweed.	Pilings.	Dredging.	Plankton.
			52	52	52	52	56	30	42	
Ophiuroidea.										
Amphipholus squamata	N.						2		12	
Ophioderma brevispina	S.					5				
Echinoidea.										
Arbacia punctulata	S.			6			18	2	17	↘
Echinarachnius parma	N.						1		12	
Strongylocentrotus drœbachiensis	N.									
Holothuroidea.										
Leptosynapta inhærens	S.		11	24		10				
Thyone briareus	S.		25			6			2	
ANNELIDA.										
Archiannelida.										
Dinophilus sp.?										↘
Polychæta										
Ampharete setosa	S.									
Amphitrite attenuata							1	9		
Amphitrite brunnea	N.							1		
Amphitrite ornata	S.		19	8		3	3 under		2	
Arabella opalina	S.		8	17	2	5	1 under	1	7	
Arenicola cristata	S.		3	6			1 under			
Autolytus cornutus	N.						1	9		
Autolytus varians	S.							1		
Chætopterus pergamentaceus	S.					1				
Cirratulus grandis	S.		11	6	1		5 under		1	
Cirratulus tenuis									1	
Capitella sp.?										
Clymenella torquata	M.		17	25		6	6 under			
Diopatra cuprea	S.		6	14		2	1 under		2	
Drilonereis longa			1	7	1					
Enoplobranchus sanguineus	M.		3							
Glycera americana or dibranchiata	S. M. }		20	53	1	12	1 under			
Harmothœ imbricata	N.		2	5	1	3	26	27	11	

Species.	Range.	Unclassified.	Mud.	Sand.	Gravel.	Eel Grass.	Rocks and Rockweed.	Pilings.	Dredging.	Plankton.
							No. Teams Reporting.			
			52	52	52	52	56	30	42	
Polychæta Con't.										
Hydroides hexagonus	S.	5	2	3	1	3	39	17	22	
Laonice viridis	S.	3	3	1						
Lepidametria commensalis	S.						1 among			
Lepidonotus squamatus	N.	1	3	5	2	2	37	29	22	
Lepræa rubra	S.				1		4 under			
Lumbrinereis hebes										
Lumbrinereis tenuis	S.		7	19	2	1	3 under		14	•
Maldane urceolata	S.		3	9		2				
Marphysa leidyi			1						3	
Nereis limbata	S.		12	9	2	3	8	3		
Nereis pelagica	N.		4	6			9	29	22	} sp.?
Nereis virens	N.		17	20		9	3 under	1		
Nicolea simplex			1	3		2	7	13	7	
Pectinaria gouldi	S.		9	18	1	5				
Potamilla sp.?									1	
Pholoë sp.?										
Phyllodoce catenula	N.	1	3	6		3	5	17	13	
Pista palmata	S.		3	1		1				
Platynereis megalops	S.					1		1		
Podarka obscura	S.		2			4	4	7	6	
Polycirrus eximeus	S.		10	14		4	15	6	12	
Polydora sp.								2		
Sabella microphthalmia	S.		1	1	2		3	7	1	
Sabellaria vulgaris	S.	1	2	2		5	6	2	4	
Scoloplos acutus			12	1		6				
Scoloplos fragilis	S.		8	19		9	1 under			
Scoloplos robustus	S.		8	10		3	1 under			
Spio (setosa?)				1						
Spirorbis spirorbis	N.		3	4	2	11	26	19		
Spirorbis tubæformis	S.						1		1	
Sthenelais leidyi	S.		6	5		4	3	4	6	
Terebellides strœmi	N.									
Thelepus cincinnatus	N.						1	3		
Trophonia affinis	S.		4	1			.		6	
Chætognatha and Sipunculoidea										
Phascolosoma gouldii	M.		13	22		3	2 under			
Sagitta sp.?										✓

Species.	Range.	Unclassified.	Mud.	Sand.	Gravel.	Eel Grass.	Rocks and Rockweed.	Pilings.	Dredging.	Plankton.
No. Teams Reporting.			52	52	52	52	56	30	42	
BRYOZOA.										
Ætea anguina	N.					5	10	12		
Alcyonidium sp.?							3			
Bicellaria ciliata	M.							3	3	
Bowerbankia gracilis	N.						3	1		
Bugula cucullifera	N.							1		
Bugula flabellata	N.						1	1		
Bugula turrita	S.	1	2			17	17	31	14	↘
Cribrillina punctata	N.								1	
Crisia eburnea	N.	1	1			4	21	18	12	
Flustrella hispida	N.					1	22			
Hippothoa divaricata	C.							1		
Hippothoa hyalina	C.							1		
Lepralia pallasiana	N.									
Lepralia pertusa	S.	1		1		10	14	6	6	
Lepralia serrata	M.							1		
Lichenopora verrucaria	N.							2		
Membranipora monostachys								1		
Membranipora pilosa	N.			1	1	6	21	13	5	
Membranipora tenuis									6	
Microporella ciliata	C.						1	2	3	
Schizoporella biaperta									5	
Schizoporella unicornis	S.	2	3		1	8	32	25	11	
Smittia trispinosa nitida	M.					1	3		15	
ARTHROPODA.										
Phyllopoda.										
Evadne nordmanni	M.									↘
Podon leuckarti	M.									↘
Cirripedia.										
Balanus balanoides	M.		5	4	3	2	48		2	↘
Balanus eburneus	S.		1	3	1	6	18	19	17	
Lepas anatifera	C.	1					1			
Arthrostraca.										
Amphithœ rubricata	N.		1	1			6	6	8	
Autonœ (Lembos) smithi	L.			1			5	5	9	
Æginella longicornis	N.						1	4		
*Caprella geometrica	S.			1		6	13	26	1	↘
Chiridotea cæca	S.		2	2		2	2			
Corophium cylindricum								2		
Cyathura carinata	N.			1		1				
Edotea triloba	M.	1	3				2	2		

* In part *Æginella*

Species.	Range.	Unclassified.	Mud.	Sand.	Gravel.	Eel Grass.	Rocks and Rockweed.	Pilings.	Dredging.	Plankton.
			52	52	52	52	56	30	42	
Arthrostraca Con't.										
Erichsonella filiformis	N.			1		2	2	2	4	
Gammarus (Sev. sp.)		2	8	11	3	15	10	28	18	✓
Haustorius arenarius	S.			2						
Idothea baltica	M.		5	4		20	9	15		✓
Idothea metallica	S.		3		2	5				✓
Idothea phosphorea	N.						1			
Jæra marina	N.	2		2	1	4	3			
Ligyda oceanica	N.						3			
Orchestia agilis	S.	10	1	33			8	2		
Sphæroma quadridentatum	S.						5			
Talorchestia longicornis	S.		2	14	6	1				
Tanais cavolinii	N.							1		
Unicola irrorata	N.								1	
Thoracostraca.										
Callianassa stimpsoni	S.		1				1 among			
Callinectes sapidus	S.		1	1						
Cancer borealis	N.					3				
Cancer irroratus	M.		5	7	2	4	6	2 near	2	
Crangon vulgaris (Crago septemspinosus)	M.		3	4	4	16	3		1	
Carcinides mænas	S.		16	4	4	4	8		5	
Heterocrypta granulata	S.								1	
Heteromysis formosa	N.	1	3	3		1	1		12	✓
Hippa (Emerita) talpoida	S.									
Hyas coarctatus	N.									
Libinia dubia	S.		14	8	1	10	3	20	16	
Libinia emarginata	S.		14	4	1	7	1	10	9	
Lysiosquilla armata										
Mysis stenolepis	M.									
Michtheimysis mixta										
Ovalipes ocellatus	S.		1	3		3	2		1	
Pagurus acadianus	M.			1					2	
Pagurus longicarpus	S.	3	19	16	5	10	7	2	22	
Pagurus pollicaris	S.	1	4	10	4	7	1	1	10	
Palæmonetes vulgaris	S.		9	7		16	8			
Panopeus sayi or Neopanope texana sayi }	S.	2	17	12	3	12	7	27	16	
Pelia mutica	S.			1						
Pinnixa chætopterana			2			2	1 among			
Pinnixa sayana (cylindrica)										
Pinnotheres maculatus	S.		3					6		
Portunus sayi	S.									
Squilla empusa	S.									

Species.	Range.	Unclassified.	Mud.	Sand.	Gravel.	Eel Grass.	Rocks and Rockweed.	Pilings.	Dredging.	Plankton.
No. Teams Reporting.			52	52	52	52	56	30	42	
Thoracostraca Con't.										
Uca pugilator	S.		21	4	3	2	8			
Uca pugnax	S.			2						
Virbius (Hippolyte) zostericola	S.	1	4	2			25	1		
Arachnoidea.										
Anoplodactylus lentus	N.	2					4	10	2	
Pallene empusa	S.					2	1	5		√
Limulus polyphemus	S.	1	12	10	2		6	1		
Tanystylum orbiculare	S.							10	1	
MOLLUSCA. *Amphineura.*										
Chætopleura apiculata	S.	3					19		16	
Gasteropoda.										
Acmæa testudinalis	N.	2	1	2		1	26	1	3	
Bittium alternatum	M.	1	6	6	2	20	17	11	8	
Busycon canaliculatum	S.			7	1		2			
Busycon carica	S.			1	1		1	1		
Cæcum pulchellum							2			
Cerithiopsis emersonii	S.	1				2	3		6	
Cerithiopsis greenii	S.					2	1 sp.?		2	
Cerithiopsis terebralis									2	
Columbella avara	S.	1	3	5	1	9	13	14	19	
Columbella lunata	S.	1	3	7		12	20	30	21	
Coryphella gymnota	N.	1			1	1	2	15		
Crepidula convexa	S.	4	12	10	1	6	20	4	7	
Crepidula fornicata	S.	2	6	15	1	2	21	12	9	
Crepidula hlana	S.	3	7	16	2	2	15	14	15	
Doris bifida	N.							1	1	
Elysia chlorotica	M.					4	1			
Eupleura caudata	S.		1	3	1		4		5	
Lacuna vincta	S.		4	4	2	14	13	21	9	
Littorina irrorata	S.						2			
Littorina litorea	N.	1	15	8	6	18	29	27		
Littorina palliata	N.		4	5	3	11	24	7	1	
Littorina rudis	N.		8	4	2	12	32	6	1	
Melampus lineatus	S.		14	4	2					
Nassa obsoleta	S.		35	14		3	9			
Nassa trivittata	S.		13	16	1	3	8	11	3	
Nassa vibex	S.		1							
Natica duplicata	S.		2	6	1	3	1 among			
Natica heros	M.		2	1	1		2			

Species.	Range.	Unclassified.	Mud.	Sand.	Gravel.	Eel Grass.	Rocks and Rockweed.	Pilings.	Dredging.	Plankton.
			52	52	52	52	56	30	42	
Gasteropoda Con't.										
Natica immaculata	N.									2
Natica pusilla	S.		1			1				
Odostomia sp.?		1	2	1		7	24	2		
Rissoa minuta	N.		3							
Rissoa sp.?										
Scalaria sp.?			1	1						
Purpura lapillus	N.			1			10			
Urosalpinx cinereus	S.	4	5	8	1	5	31	22	5	
Pelecypoda.										
Anomia aculeata	N.					1	12	4	5	
Anomia ephippium	S.		2			1	22	10	7	
Arca pexata	S.			1			5	1	7	
Arca ponderosa	S.									
Arca transversa	S.		1	1			1		4	
Astarte castanea	M.								3	
Astarte undata	N.								5	
Cardium pinnulatum	N.								5	
Clidiophora trilineata	S.								3	
Corbula contracta	S.			2						
Cumingia tellinoides	S.		10	11		6	1			↙
Ensis directus	M.		9	9		2	5 among			
Gastranella tumida	S.							1		
Gemma gemma	N.			3			1			
Lævicardium mortoni	S.		11	5	2	4	1 among			
Lyonsia hyalina	S.	1		2						
Macoma tenta	S.		2	2		1			1	
Mactra lateralis	S.			1						
Mactra solidissima	N.			3			2		2	
Modiolus demissus	S.		22	15	3	6	8			
Modiolus modiolus	N.		10	7		4	6	3	3	
Mya arenaria	N.		23	21	3	6	5 among			
Mytilus edulis	N.		11	11	5	4	24	28	7	
Nucula delphinodonta	N.								1	
Nucula proxima	M.		3	3	1	4	4 among		3	
Ostrea virginica	S.		10	5			9			
Pecten irradians	S.		9	9		11	4		4	
Petricola pholadiformis	S.		3	2	3		1			
Saxicava arctica	N.						2			
Solemya velum	M.		10	20		4	4 among			
Tellina tenera	S.		5	16	3	4	1			
Teredo navalis	N.	19		2	1	1	1	7		

Species.	Range.	Unclassified.	Mud.	Sand.	Gravel.	Eel Grass.	Rocks and Rockweed.	Pilings.	Derdging.	Plankton.
							No. Teams Reporting.			
			52	52	52	52	56	30	42	
Pelecypoda Con't.										
Venus mercenaria	S.	1	19	14	2	1	4 among		2	
Yoldia limatula	N.									
Zirphœa crispata	N.		1							
Cephalopoda.										
Loligo pealii	S.				3					√
CHORDATA.										
Appendicularia longicauda	M.									√
Amarœcium constellatum	N.	3				2	3	19	29	14
Amarœcium pellucidum	S.									12
Amarœcium stellatum	S.									
Botryllus schlosseri	N.	1					13	7	5	√
Didemnum lutarium	N.	2					6	13	30	
Dolichoglossus kowalevskyi	S.			12	14	6	2	3 under		
Molgula manhattensis	S.	2		3		1	6	8	24	1
Molgula papillosa	N.							1		
Molgula arenata	S.							1		
Perophora viridis	S.	2			1		5	16	28	4
Styela partita	S.			1	1		6	17	29	9

IV.

The entire mass of data available was analyzed in 1917 and again in 1920 in an attempt to discover the relationships existing between animal associations from the different types of environments. The first type of analysis was planned to discover the number of species common to different combinations of these habitats and conversely to find the number of animals peculiar to each type of habitat. The analysis was not repeated after the 1921 records were available because it was not considered that these records would materially change conclusions already arrived at by the preceding work.

The analysis of the records from 1915–1920 inclusive follows:

Habitats.	Number of Species.
Flats, rocks, pilings and dredgings	54
Flats, rocks and pilings	16
Rocks, pilings and dredging	10
Flats, pilings and dredging	3
Flats, rocks and dredging	9
Flats and under or on rocks	26
Wharfs and rocks	8
Flats and rocks	11
Dredging and rocks	5
Flats and pilings	2
Pilings and dredging	2
Flats and dredging	9
Flats, under rocks and dredging	4
On or under rocks	10
Pilings	7
Dredging	13
Only under rocks	' 1
Flats	41

The analysis shows that at the close of the 1920 season 54 species had been recorded from all four types of habitats studied while only 16 species were limited to and found in all of the three habitats excluding dredging. The flats have the greatest number of peculiar species, with 41, and but few forms are limited to any one of the other habitats.

The same material analyzed in another fashion is shown in Table I. Here the attempt is to show the total numbers of

TABLE I.

	Wharfs.	Flats.	Rocks.		Dredging.
Wharfs	110 100%	83 74%	80 73%	76 69%	(26 only in Harbor)
Flats	83 44%	187 100%	80[1] 43%	87 46%	(28 only in Harbor)
Rocks	80 72%	80 72%	111 100%	81 73%	(22 only in Harbor)
Dredging	76 64%	87 73%	81 69%	119 100%	(32 only in Harbor)

animals found in each of the four major divisions of the littoral zones of the region in comparison with each of the other divisions.

[1] 30 more dug occasionally among or under rocks.

Again the analysis follows the records through the season of 1920. The comparison is based on total distribution records as was the last. That is, for the purposes of this table the finding of a given animal once in a given type of habitat is as significant as though it were abundant there. There were 242 species in the catalogue when this study was made.

It is immediately apparent that this type of analysis serves only to call attention to the larger number of animals taken from the flats and beyond indicating the higher specificity of the flats, shows no evidence of relationships that may exist between different habitats. The species taken from any given type of habitat are found to be approximately equally distributed in the other habitats of the region. Such experience has led casual observers to conclude that relationships between different animal associations can not be analyzed.

In order to make such an analysis, the records were studied from another angle. Species approximately equally distributed through the different associations and those reported for one season only were eliminated. Then the remaining species were listed according to the habitat in which they are most abundant. When a species was found equally abundant in two habitats it was listed from both. The association in which the animal was next most abundant was also estimated. Unfortunately these records are based on "experience" cards that seldom give specific figures and on the number of collecting teams that have reported the species from the different associations, as in the data given in the check list, rather than on strictly quantitative grounds and while substantially correct, they lack the finality that statistical treatment would give.

This type of analysis gives a real basis for comparisons of the relationship between the different associations. It is of interest that the relationships shown by Table II. are practically the same as given by an analysis of the entire catalog in 1917. In other words, the early collecting gave the typical forms characteristic of the environment while much of the later work has yielded in addition to these characteristic species, a number of accidental or incidental records.

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TABLE II.

RELATION OF DIFFERENT HABITATS BASED ON DISTRIBUTION OF CHARACTERISTIC ANIMALS.

Name of Association.	No. Species.	Peculiar Species.		Species Found Next Most Abundant in:								
				Pilings.		Rocks.		Flats.		Dredging.		Under or Among Rocks.	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Wharf pilings......	32	2	6	—	—	24.5[1]	77	2.5	8	2.0	6	0	0
Rocks and rockweed	32	3	9	14.5	45	—	—	13	41	1.5	5	0	0
Dredging.........	20	3	15	3	15	9	45	3	15	—	—	0	0
Flats............	40	22	25	2.5	3	15	17	—	—	17.5	20	30	33

The association of the wharf pilings is found to be closely related with that of the rocks; 77 per cent. of the animals common in the former are next most abundant in the latter. This fits with one's general impression that the two sets of animals are much the same but with the emphasis placed on different species so far as numbers are concerned.

The animals common on the rocks are next most abundant on the pilings but almost as many are nearly as abundant on the flats. The latter is to be expected from the fact that the rocks extend up from the flats, often forming a belt only a few feet wide in the intertidal portion of the flats, and that single rocks frequently occur surrounded by typical mud or sand flats, and from the further consideration that the eel grass offers almost as good a place of attachment for many animals as do the rocks.

The animals taken commonly in dredging are more closely related to the rock association than to the others. This is because our dredging operations have been done on clean hard bottom where the water conditions are similar to those found among rocks. Dredging in mud as in Buzzards Bay would give different results and transition bottom associations are indicated from the dredging work in Great Harbor.

Again the marked independence of the flats as a special habitat is shown by the larger number of animals frequently taken there

[1] Where animals are approximately equally distributed between two different associations they are summarized by fractional representation.

in some abunbance and by the larger number of peculiar species. They are not closely related to other habitats if one excepts the conditions under the rocks which scarcely form a different association. Its main difference is that burrowing and exposure are more limited than on the open flats. The animals common on the flats are particularly absent from wharf pilings showing, as would be expected, that these habitats have little in common.

If quantitative data were available, the distinctions here found would doubtless stand forth more plainly. The fact that a beginning can be made in indicating relationships by the methods used when no relationships appeared from an analysis of bare check lists emphasizes the need of quantitative studies in animal ecology.

This need was first recognized by Forbes (1907) who devised a mathematical formula for determining the existence of an association. In 1911, as the result of studying seasonal succession in ponds, I concluded that qualitative work gives insufficient basis for exact conclusions. Shelford in 1915 repeats the formula of Forbes, and Michael (16, 21) has done more than anyone else in America in showing the fundamental need of quantitative investigations in ecology and in developing formulæ to enable one to study associations on a quantitative basis.

The work here presented is of course only quasi-quantitative in character but the greater clearness obtained indicates that much of the muddle of animal ecology may be cleared by the further development and the application of quantitative methods in field researches. The problem of the ecologist studying littoral distribution is not so hard as that of the plankton student where as Michael says (1921): "Granting the equivalent of the oak tree or pine tree association, the marine ecologist finds difficulty not only in describing it but even in finding it. Since he cannot directly witness such an association, he is compelled to rely on indirect evidence furnished by tow-net or similar apparatus. In other words his only recourse is to measured magnitudes and application of mathematical logic thereto." For the exact determination of such relations the methods here used are almost as gross as are the ordinary qualitative observations in trying to solve the relationships existing between littoral associations.

It is true that analysis of the results of preliminary collecting showed the same relations as the quasi-quantitative analysis of more complete records in the Woods Hole region.   Unfortunately one cannot be sure that the animals found in such preliminary work are really the typical animals since they may obviously contain many incidental forms.   In other words in a random sample one is more apt to collect animals typical of the habitat than incidental forms but he can never be sure of this without further work.

V. The Effect of Continued Collecting on Distribution Records.

In 1917 when the collecting records were first studied seriously there were 181 species in the catalog.   In 1920 when a similar study was made, the catalog listed 242 species.   In the interim the Sound Gutters, Lackey's Bay and Great Harbor had been added to the localities visited.

In 1917, 11 species were recorded only from the wharf pilings. The later lists show 7 species so limited but this includes only one (*Tetrastemma*) of the previous list.   In 1917 two species were recorded only from rocks or rockweeds, while on the later list there were 10 such species including only *Clava leptostyla* from the preceding list.   In the first comparison there were 10 animals recorded only from dredging; in the later one, 13, which includes four of those on the preceding list: *Arca ponderosa*, *Strongylocentrotus droebachiensis*, *Heterocrypta granulata*, and *Amaroecium stellatum*.   .

On the 1917 list, 71 species were recorded from the flats only. After four more years' work this had shrunk to 41 providing animals found in the sand under and among rocks are excluded. Of these only 17 appear on both lists.   They are: *Edwardsia elegans*, *Eloactis producta*, *Bdelloura candida*, *Syncoelidium pellucidium*, *Ophioderma brevispina*, *Chaetopterus pergamentaceous*, *Platynereis megalops*, *Scoloplos acutus*, *S. robustus*, *Spio* (*setosa?*), *Callianassa stimpsoni*, *Mysis stenolepis*, *Squilla empusa*, *Melampus lineatus*, *Clidiophora trilineata*, *Pecten irradians*, and *Tellina tenera*.   .

On the earlier lists, fifteen species were recorded from some place in each of the four main types of habitats: wharf pilings, rocks, flats, and dredging.   In 1920 this list had increased to 54. These results mean, as has already been suggested, that as collecting has proceeded, animals have been picked up in habitats in which they are not abundant.   The scarcity of many of these is shown by the number of single specimen records on the lists. There is little doubt but that if the present type of collecting were continued long enough, there would finally be stray records of many of the animals found in the region from each type of habitat.   Even dredging, which we have usually carried on in deep water in Vineyard Sound, yields a different type of animals and becomes more closely related to other habitats as a result of dredging records taken in Great Harbor.   In some one or more of their dredging operations, the *Biological Survey* found most of the animals we have taken from inshore digging.   This result might be expected from the fact that some of their dredgings are recorded from less than 10 feet of water.   If made at high tide, these would be almost as close inshore as our deepest collecting on wading and digging expeditions when we often collect out to four feet of water at low spring tides.

In other words, in such a small region as we are now considering, provided with strong tidal currents which aid in distribution, the animals tend to become widely distributed and occasional specimens will be found that can tolerate for a time conditions that are essentially unfavorable.   Under these conditions the mere record of the presence of a species in a given habitat means very little unless there is due consideration of its abundance and duration in that locality.   One is thus driven again to the conclusion of the last section, that quantitative work is necessary before final judgment can be passed in the matter of the constitution of animal associations.

We have found no evidence that the long continued collecting over the same grounds by the Invertebrate Class, nor the commercial collecting of the Supply Department of the M. B. L. has affected the number of animals present within the past nine years sufficiently for the effect to be noticeable by the collecting methods we have used.   With growing experience in collecting

each year we have broken previous records with monotonous regularity, for numbers of species from most of the localities we visit. This could not have continued so long had the animals been becoming less abundant.

The number of animals present in a given locality must depend more on the availability of suitable breeding places and abundance of food than upon such disturbing influences as summer collecting, particularly when the collecting does not reach all the breeding habitats of a region and there is adequate means of distributing young stages. This conclusion is emphasized by the rapid recovery in numbers of *Arbacia* after their almost complete disappearance following the winter of 1917–18 (Allee, '19) and in the face of their destruction by the thousands in the research work carried on in the Woods Hole laboratories.

VI. SUMMARY.

1. Analysis of distribution records in the four major types of habitats of the Woods Hole littoral, viz., wharf pilings, rocks and rockweeds, flats, and the sea bottom in deeper water show that mere records of species present in the different habitats fail to indicate any relationship between the different types of associations.

2. By eliminating species known to be approximately equally distributed throughout and records for one year, only, and classifying the remaining species in terms of places where they are most abundant and next most abundant one finds:

(*a*) The association of the wharf pilings is closely related to that of the rocks.

(*b*) Species taken in dredging on clean hard bottom are found in next abundance on the rocks.

(*c*) The associations of the flats are highly independent of the others in the region but continue in the mud and sand under and around rocks.

(*d*) That some degree of quantitative· work is necessary in order to determine the relationships of animal associations.

3. Preliminary collecting in a region tends to give the obvious forms and gives similar results in analysis to the type of quasi-qualitative work described in this report.

4. The number of animals present in the Woods Hole region has not been noticeably affected by the intensive collecting carried on there during the nine years covered by these studies.

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FOOD AND PARTHENOGENETIC REPRODUCTION AS RELATED TO THE CONSTITUTIONAL VIGOR OF *HYDATINA SENTA*.[1]

W. V. LAMBERT, W. S. RICE, AND H. C. A. WALKER.

There has been a general belief for many years that cross-fertilization is beneficial to a race. Darwin studied the effects of inbreeding and crossbreeding in many plants and came to the general conclusion that while inbreeding has a deleterious effect, crossbreeding has a beneficial effect upon the races involved.

In experiments with infusoria Calkins has found that if conjugation is prevented the race gradually weakened and finally died. He observed, however, that by the artificial stimuli of various chemicals in the culture water he was able to prolong the life of one race of *Paramecium caudautam* to the 742d generation.

Investigations in regard to the effects of parthenogenetic reproduction have been carried on by Shull and Whitney with the rotifer, *Hydatina senta*. Both of these investigators have found that continued parthenogenesis in pedigree races of rotifers has resulted in a gradual weakening and loss of vigor, and in some experiments death. In Whitney's experiments one race of rotifers died out from general exhaustion in the 384th generation and another in the 546th generation. A third race, discontinued in the 443d parthenogenetic generation had also shown a marked decrease in constitutional vigor.

Investigations of other workers, however, do not seem to confirm these general results. Woodruff, in his observations on the *Paramecium aurelia*, found that a race of these animals kept in cultures made from natural pond waters did not undergo marked depression or physiological changes. This race at the end of eight years, after having passed through 5250 generations without conjugation, was in a normal condition. Another race, however, subjected to the relatively constant hay infusion cultures generally employed, died after passing through several hundred generations.

[1] Studies from the Zoölogical Laboratory, The University of Nebraska, No. 134.

King, also, in experiments with albino rats has found that continued inbreeding of the rat, when subjected to a varied and well balanced diet, does not weaken the race. On the contrary, the strains were larger, more fertile, and lived longer than many strains of stock rats in which no inbreeding was allowed. Castle, also, found after inbreeding the fly *Drosophila* (brother and sister matings) for fifty-nine generations, that no apparent decrease in the size and vigor, as compared to those with which he started, was noticeable. Noyes has recently finished the 250th partheno-genetic generation of the rotifer *Proales decipiens* and has detected no noticeable decline in vigor. Moreover, males of this species have never been seen. East and Jones are inclined to believe that if proper changes in the diet had been made in the experi-ments of Whitney and Shull upon the rotifer *Hydatina senta* that the normal vigor of the races would have been maintained.

PROBLEM.

The following experiments with the rotifer, *Hydatina senta*, were begun in order to determine if possible whether the gradual loss of vigor is due to the parthenogenetic reproduction or to a too restricted diet. The various races used in these experiments were kept under identical conditions as to temperature, light and culture water. Race *A* was fed a colorless *Polytoma* diet, whereas race *B* was fed a diet consisting of the *Polytoma* plus several kinds of green protozoa, predominately *Chlamydomonas*. The plan has been to keep the races in a parallel series and to study the effect of the two diets upon the two races. A third race (*C*) which has been obtained from wild rotifer cultures in the natural environment has been introduced into the series in order that the rate of reproduction of the rotifers under restricted and controlled environmental conditions might be compared with the rate of reproduction of those under the uncontrolled conditions of nature.

Acknowledgment is due Professor David D. Whitney for his suggestions and supervision of the work.

METHODS.

Some dried fertilized eggs of the rotifer, *Hydatina senta*, were collected at Lincoln, Nebraska, on September 17, 1920. One of

these eggs hatched on September 29, and on October 1 two parthenogenetic sisters were isolated. One became the mother of what has been called race A and the other became the mother of what has been called race B. The two sister parthenogenetic races A and B were kept in syracuse watch glasses. Usually once in forty-eight hours five daughter-females of each race were isolated, each daughter-female being placed in a separate watch glass. They produced the young females of the succeeding generation. Race A was fed on a pure culture of the flagellate, *Polytoma*. This food was grown in horse manure solutions, which was prepared as follows: eight hundred cubic centimeters of fresh horse manure and one thousand cubic centimeters of tap water steam sterilized for one hour. This was cooled and diluted in the proportion of one part horse manure solution to two parts of boiled rain water. *Polytoma* were then put into it in an open pan and placed at about 18° C. The *Polytoma* grew very quickly and in 24–48 hours immense numbers of them were produced. This culture, ranging from 24–96 hours old, was decanted and replenished daily—one part culture medium to two parts boiled rain water being added. From the surface of this culture the *Polytoma* were taken with a sterilized pipette. This food was centrifuged so as to make it concentrated enough so that one drop was sufficient for each watch glass containing a female rotifer and a small amount of filtered rain water.

Race B was fed both from the pure culture of *Polytoma* and from two or three cultures of green flage·lates, *Chlamydomonas*, *Chloroganium euchlorum*, and *Euglena*.

As some of the green food grew in salty cultures it was always washed with rain water before being placed in the watch glasses as food for the rotifers.

During the whole period of fifteen months the two races have been conducted in parallel generations. The external factors and environment have been as near alike as it has· been possible to make them. The individuals of each generation were isolated at the same time, were put into the same kind of watch glasses and with approximately the same amount of filtered tap water, the only difference being in the food. The watch glasses were stacked side by side. During the day the room was lighted by the north light.

The method used for deciding whether the races had maintained their original vitality or whether they had decreased in their constitutional vigor has been the rate of parthenogenetic reproduction. In order to determine the comparative vigor of the races *A* and *B*, their rates of parthenogenetic reproduction were obtained by counting the young produced by a mother during a certain period of time for several successive generations at intervals of about thirty days.

The first comparison was made by counting the young daughter-females of both the races *A* and *B* from generations 22–29. The second comparison was made by counting the young in the same manner as above from the generations 43–47. The third was made by counting the young from generations 61–67. No green food was given to race *B* during the generations when these counts were made so that conditions would be identical at the time the rate of parthenogenetic reproduction was being determined.

During the above named generations fifteen daughter-females of approximately the same size of each race were isolated. This was done in order to give a greater number from which to get the average of young females produced, thereby decreasing the chance of error.

When the third and also the last comparisons were made fifteen females from various wild races of *Hydatina senta* were isolated and placed in separate watch glasses. These wild females have been called race *C*. This race was fed only pure culture of *Polytoma*. It was kept for only ,a few generations, being conducted under identical conditions and parallel to generations 65–67 and 169–175 of races *A* and *B*. This introduction was made in order to make a comparison of the rate of parthenogenetic reproduction between races *A* and *B* and various wild races taken from their normal environment.

RESULTS.

The first series of observations on the rates of parthenogenetic reproduction in these two races was started at the beginning of the twenty-second generation. The results at this time as can be seen in Table I., are extremely variable. Only five daughter-

TABLE I.

No. of Partheno- genetic Generation.	Date of Isolation.	No. of Young Females Iso- lated and Reproducing.	Period of Growth and Reproduction.	Race A. Av. No. of Daughters Produced.	Race B. Av. No. of Daughters Produced.
22........	11/16/20	5	48 hrs.	9.22	10.25
23........	11/18/20	5	48	7.20	5
24........	11/20/20	5	48	4	1
25........	11/22/20	5	48	7.8	1.4
26........	11/24/20	5	48	1.5	1.5
27........	11/26/20	5	72	4.2	4.5
28........	11/29/20	5	72	13.8	15.66
29........	12/2 /20	5	48	7	7.2
Average ..		5	54 +	6.84	5.81

females were isolated every other day for making the counts on the two races. The average number of young daughter-females produced in each forty-eight hour period at this time was extremely variable. The mothers of the A race during this period produced an average of 6.84 daughter-females while those of the B race produced an average of only 5.81.

The two parthenogenetic races were then carried on parallel with each other until the beginning of the forty-third generation at which time another series of observations was started on the number of young daughter-females each mother would produce in a given length of time, approximately 48 hours. As new generations were isolated great care was taken to pick uniform-sized daughters. One investigator did all of the isolating during this series of counts in order to insure the greatest uniformity. Considerable difficulty was experienced during this series of observations because of the large per cent. of male-producing females. These had to be discarded for only the mothers producing daughter-females were considered in calculating the averages.

The A race during this second series of observations produced an average of 7.58 daughter-females while the B race produced 8.88. Considerable variation occurred throughout the different generations, but the variations were less in this instance than they were in the preceding series of observations. A complete record of the results of this observation can be seen in Table II.

The two races were then carried on for fourteen generations before another series of observations was begun. At the begin-

TABLE II.

No. of Partheno-genetic Generation.	Date of Isolation.	No. of Young Females Iso-lated and Reproducing.		Period of Growth and Reproduction.	Race A. Av. No. of Daughters Produced.	Race B. Av. No. of Daughters Produced.
		A.	B.			
43..........	1/ 6/21	5	5	48 hrs.	7.4	10
44..........	1/ 8/21	10	11	48	6.6	3.8
45..........	1/11/21	14	13	48	6	10.8
45..........	1/11/21	5	5	48	6.5	6.9
46..........	1/13/21	8	11	48	12	15.2
47..........	1/15/21	15	14	52	7	6.6
Average.....				48.7	7.58	8.88

ning of the 61st generation fifteen individuals from each race were isolated as before and placed in separate watch glasses,— one person doing all the isolating as in the preceding series. No male-producing females appeared in this series of observations and the number of young produced in a given length of time was more nearly uniform throughout than was the case in the two preceding observations. The average number of young produced by the A race in the last three generations of this series of counts was 2.66 in an average of 46.3 hours. In the B race for the three generations an average of 3.68 daughter-females were produced in the same period of time.

TABLE III.

No. of Partheno-genetic Genera-tion.	Date of Isolation.	No. of Young Females Isolated and Reproducing.			Period of Growth and Repro-duction.	Av. No. of Daughter-Females Produced.		
		A.	B.	C.		Race A.	Race B.	Race C.
61........	2/13/21	15	13		48 hrs.	4.84	5.84	
62........	2/15/21	15	15		41	4.64	5.44	
63........	2/17/21	15	15		47	2.90	3.37	
64........	2/19/21	15	15		48	4.25	4.37	
65........	2/21/21	13	15	15	44	1.15	2	2.33
66........	2/23/21	15	14	15	48	3.56	4.21	4
67........	2/25/21	5	14	14	48	3.80	5.21	4.25
Average..					46.3	2.66	3.68	3.53

At the beginning of the sixty-fifth generation, counts on the

wild race of rotifers, race C, were started. They were reared under identical conditions with the A and B races and the counts were carried over a period of three generations. The average number of young produced by these mothers in 46.3 hours was 3.53. A complete record of the results of this observation can be seen in Table III.

The two parthenogenetic races were then carried on parallel with one another until the beginning of the 79th generation at which time a fourth series of observations was started.

During this fourth series of observations the A race produced an average of 7.46 daughter-females while the B race produced 8.29. Very little variation occurred throughout the different generations during this series of observations. A complete record of the results of this observation is given in Table IV.   ·

TABLE IV.

No. of Par- thenogenetic Generation.	Date of Isolation.	No. of Young Females Isolated and Reproducing.		Period of Growth and Reproduction.	Av. No. of Daughter- Females Produced.	
		A.	B.		Race A.	Race B.
79..........	3/22/21	15	15	48 hrs.	10.06	11.46
80..........	3/24/21	15	15	48	7.06	7.53
81..........	3/26/21	13	14	54	3.92	4.28
82..........	3/28/21	15	15	46	6.53	7.26
83..........	3/30/21	15	15	48	9.73	10.93
Average.....				48.8	7.46	8.29

The two races were then carried on for thirteen generations before another series of observations was made. The average number of hours between counts in this series of observations was 65.33 hours, the increase in length of time being due to the slowness of reproduction which was probably caused by the low temperature of the laboratory. The average number of young daughter-females produced during this series by race A was 3.32, while the number by race B was 5.15. A complete record of the results of this series is given in Table V.

The two races were again carried on parallel with one another until the beginning of the 106th generation. At this time they showed a very great degree of uniformity between the different

TABLE V.

No. of Parthenogenetic Generation.	Date of Isolation.	No. of Young Females Isolated and Reproducing.		Period of Growth and Reproduction.	Av. No. of Daughter-Females Produced.	
		A.	B.		Race A.	Race B.
96..........	4/28/21	14	15	77 hrs.	4.13	6.53
97..........	5/ 1/21	13	15	70	2.23	4.33
98..........	5/ 4/21	15	15	50	3.60	4.60
Average.....				65.33	3.32	5.15

generations, but there was a slight decrease in the difference between the number of young daughter-females produced by the two races. The *A* race during this series of observations produced an average of 9.19 daughter-females while the *B* race produced 10.06. A complete record of the results of this observation is given in Table VI.

TABLE VI.

No. of Parthenogenetic Generation.	Date of Isolation.	No. of Young Females Isolated and Reproducing.		Period of Growth and Reproduction.	Av. No. of Daughter-Females Produced.	
		A.	B.		Race A.	Race B.
106........	5/23/21	14	13	48 hrs.	9.28	10.07
107........	5/25/21	14	14	48	8.14	10.64
108........	5/27/21	15	15	48	6.60	7.20
109........	5/29/21	15	15	48	11.46	11.46
110........	5/31/21	13	11	50	10.46	10.91
Average.....				48.4	9.19	10.06

The parthenogenetic reproduction of the parallel races was continued over the summer of 1921 without any further observations. The usual care was employed in their feeding. About the middle of August a wild race, *C*, was introduced. The seventh series of observations was begun with the 169th generation of the *A* and *B* races and the 10th of the wild race *C*. Considerable variation was found in the number of young produced by successive generations of each race. The difference, however, in the number of young of the *B* race as compared to the numbers

of young of A and C was marked.   The average period of growth and reproduction was 63.6 hours, during which period the average number of young of the A race was 6.86; of the B, 9.35; and of the C, 8.66.   The complete tabulation of results may be found in Table VII.

TABLE VII.

No. of Partheno-genetic Generation.	Date of Isolation.	Number of Young Females Isolated and Reproducing.	Period of Growth and Reproduction.	Average No. of Daughter-females.		
				$A.$	$B.$	$C.$
$\left.\begin{array}{l} A \\ B \end{array}\right\}$ 169........ C—10........	10/ 5/21	10	72 hours	9.4	14.1	14.9
$\left.\begin{array}{l} A \\ B \end{array}\right\}$ 170........ C—11........	10/ 8/21	10	66 hours	9.8	10.1	10.33
$\left.\begin{array}{l} A \\ B \end{array}\right\}$ 171........ C—12........	10/11/21	10	56 hours	6.5	9.4	7.9
$\left.\begin{array}{l} A \\ B \end{array}\right\}$ 174........ C—15........	10/22/21	10	54 hours	1	2.75	1.7
$\left.\begin{array}{l} A \\ B \end{array}\right\}$ 175........ C—16........	10/25/21	10	70 hours	7.6	10.4	8.5
Averages.......		10	63.6 hours	6.86	9.35	8.66

The three races were then carried on side by side.   Since the 145th generation the green food given Race B has been made up for the most part of *Euglena*, in place of the *Chlamydomonas* heretofore used.   About the same time as the change in the green food the centrifuging of the *Polytoma* culture was discontinued and more drops of the unconcentrated food given instead. After the seventh series of observations, the investigators began to encounter difficulty with the supply of *Polytoma*.   However, all three races of rotifers continued to. reproduce parthenogenetically, although with slightly less than usual vigor in Race A.   Nevertheless, notwithstanding all care possible in feeding, isolation and temperature the A race, fed only *Polytoma*, died out with the 198th generation, probably due to the faulty
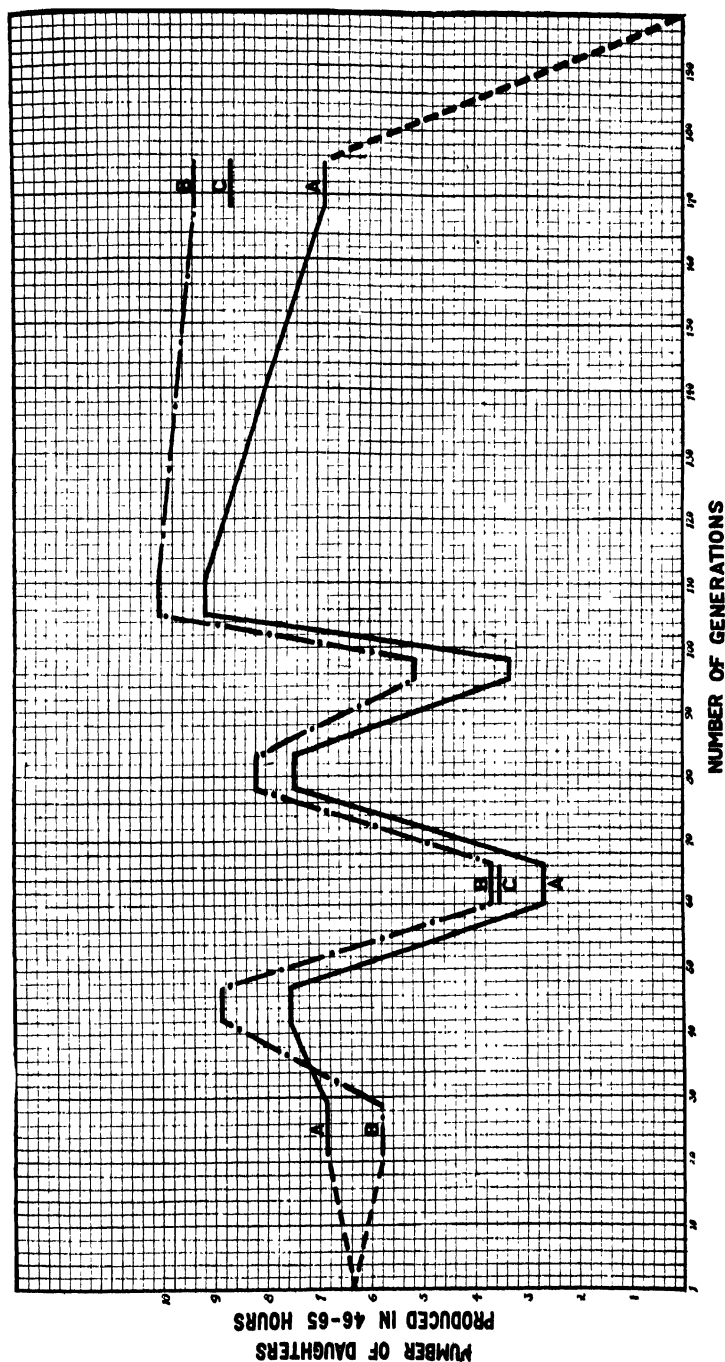
FIG. 1. Graphic representation of the results obtained from parallel races fed upon different diets and reproducing parthenogenetically. The unbroken line traces the history of Race *A*; the broken, of Race *B*; the other, of the wild races *C*, introduced at intervals for comparison. The lines from generation 1 to 22 and 176 to 198 are hypothetical, since no tests were made during these periods. The rate of reproduction of R e *A*, which was kept on the pure *Polytoma* diet, gradually declined, whereas Race *B*, kept on a mixed diet, showed no decline in the rate reproduction.

Polytoma culture. Race *B* continued to the 209th generation and *C* to the 43rd, without apparent loss of vigor, when the experiment was discontinued. The entire history of the two races, *A* and *B*, may be seen in Fig. 1. These two races started from two sisters and their rate of reproduction was determined at seven different periods ranging over about 13 months. For some unknown reason Race B showed a lower rate of reproduction at the first count, but at the second count the rate had risen above that of Race A, and furthermore, in all the subsequent counts, covering about a year, it remained above the rate of Race A. Toward the end the two rates began to diverge considerably due to the gradual weakening of Race A while Race B maintained its former rate of reproduction.

CONCLUSIONS.

From the fact that the *A* race died out before the 200th generation it is obvious that some unexpected influence must have entered, most probably poor food, since other cultures of *Hydatina senta* have reproduced four and five hundred generations parthenogenetically. Therefore, our results can hardly be called conclusive, but the opinion seems to be justified that the constitutional vigor of the *A* race, fed upon an exclusive colorless diet, was noticeably weakened, while that of the *B* race, fed upon a diet of *Polytoma* plus a green food containing carbohydrates, was sustained and even raised above that of the wild races. If this is true, as it certainly seems to be, it would appear that the decline in vigor and eventual death in the races observed by Shull and Whitney was due to an unbalanced diet, rather than to the parthenogenetic method of reproduction, as they concluded.

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BIOLOGICAL BULLETIN

STUDIES IN MARINE ECOLOGY: III.
SOME PHYSICAL FACTORS RELATED TO THE DISTRIBUTION OF LITTORAL INVERTEBRATES.

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I. PURPOSE AND SCOPE OF THE INVESTIGATION.[1]

The first two papers in the present series give the results of a general faunistic ecological survey of the Woods Hole littoral

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region which occupied the summers of 1913–1921 inclusive. The records given there show the distribution of the common littoral invertebrates and discuss the relationships of the different animal associations present on the basis of their animal components.

The present paper deals with the results of an intensive study of some of the localities considered in the preceding sections, which was carried on in August and early September of 1920, with some additional data from records taken the following summer. In addition to considering the direct effects of different types of bottom and shores, currents, tides and vegetation, this study is particularly concerned with the possible correlation of temperature, salinity, oxygen content and pH with the intertidal and upper adtidal animal associations of the region immediately around Woods Hole.

Reference to the map published in connection with Study I. of this series will give the general location of the region and the *Biological Survey* [1] (pp. 170–190) gives a good account of the general physiographie features. The more important of these for the purpose of our discussion are:

1. The whole region lies far back from the edge of the continental shelf. The 20 fathom line runs 10 miles off Martha's Vineyard while the 100 fathom line, marking the edge of the continental shelf, is 75 miles from Gayhead and about 90 miles from Woods Hole.

2. The unequal height of the tides in Buzzards Bay and Vineyard Sound combined with the time difference of tides in the two bodies cause strong currents to flow almost incessantly from one to the other. All the localities discussed in this paper are directly affected by these tidal currents except the wharf pilings at Vineyard Haven and the bay at Gansett.

3. There is a general westerly drift in the region so that the *Biological Survey* estimates that all the water in Vineyard Sound is renewed weekly and in Buzzards Bay in about double that time.

4. The temperature is influenced by the Gulf Stream although his is normally shut from shore by a wall of cold water. The water immediately around Woods Hole is warmer in summer

[1] Sumner, Osburn and Cole: A Biological Survey of the Waters of Woods Hole and Vicinity. Bul. Bur. Fish., Vol. 31.

and probably cooler in winter than outlying waters.[1] The *Biological Survey* reports an annual temperature range of 25° C. at the Fish Commission pier where the water is much mixed by the current through the Hole. The range is greater in stagnant water such as that in Blind Gutter at Lackey's Bay where the annual range is over 33° C.[2]

5. The intertidal zone is comparatively narrow, being more extensive in Buzzards Bay than in Vineyard Sound.

6. The shores of the region are usually lined with rocks which extend well below the low tide level and give way there to sandy bottoms in the more exposed places. In sheltered regions mud of organic origin is deposited over the sand.

7. The *Biological Survey* found that the salinity of Buzzards Bay averaged 3.00 per cent.; that of Vineyard Sound, 3.075 per cent. The open Atlantic runs to 3.6 per cent. or more.[3]

During the investigation the open water was sampled in four inshore localities:[4] Off the Buzzards Bay entrance to N. W. Gutter (Map, No. 10), in the Hole at Woods Hole; in Vineyard Sound at the entrance to Lackey's Bay (Map, No. 6) and at the entrance to Gansett Bay (Map, No. 11).

The wharf pilings were studied at Crane's Wharf (Map, No. 5) near the steamboat wharf in Woods Hole and at the old steamboat wharf in Vineyard Haven (Map, No. 1). The rocks at Northwest Gutter near its Bay entrance (Map, No. 10), in Lackey's Bay at the entrance to Blind Gutter Flats (Map, No. 6) and at Gansett. The associations of the flats were studied at Northwest and Blind Gutters and at Gansett, together with some observations in the creek leading to Southwest Gutter. In all 122 sets of collections were made.

These localities were chosen for intensive study because they are typical of the region and because of their proximity to and ease of access from the laboratory.

[1] Cf. discussion in Biological Survey, pp. 37 and 50.

[2] Water over the eel grass in Lackey's Bay had a temperature of 32 deg. C. on the afternoon of August 15, 1920. There is no reason to suppose that this temperature is unusual. In winter the temperature must frequently fall below the freezing point of sea water.

[3] See Murray and Hjort.

[4] For descriptions of the different habitats see Study I. of this series.

II. Methods.

The water collections for oxygen samples were usually made according to the method given in Standard Methods for Water Analysis.[1] Where this method was impracticable, as in surface collections, the sample was taken by means of a two-valve rubber bulb. In all cases more than four times the amount of water necessary to fill the sample bottle was run through it before the sample was taken.

The oxygen determinations were by the Winkler method. The chemicals were added and the titrations were made in the field.

Salinity was ascertained by the specific gravity method with standardized bulbs loaned by Dr. Mitchell of the U. S. F. C. The salinity is expressed in the tables as calculated from the specific gravity according to the data given by True (1915).

The pH was determined colorimetrically by comparison on the spot with standard colors in tubes specially prepared for salt water by Hynson Westcott and Co. Two indicators were used mainly: Thymolsulphonephthalein (thymol blue) with a color range from 8.0 to 9.6 and dibromthymolsulphonephthalein (bromthymol blue) ranging from 6.0 to 8.0. These sets of standard colors were made up in June before they were used in August.[2] Doubtful determinations in the 7.7–8.0 range were tried with both indicators and on occasion with phenolsulphone-phthalein, range 6.6–8.6. This last set of standard colors were made up in distilled water. Such readings were corrected for sea water by subtracting 0.1 from the observed reading.[3] Comparative readings with this correction applied are given in Table I.

The water for pH determinations was collected in a new 40 c.c. injecting syringe with a tight-fitting piston, either directly from the surface or from the large container of the standard apparatus used in collecting oxygen samples. This water was also used in

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[1] Report of the Committee on standard methods of water analysis. Jour. Inf. Diseases, Suppl. No. 1, pp. 1–141.

[2] The dibromthymolsulphonephthalein set was loaned me by Dr. E. B. Powers in 1920 and by Miss Myra Sampson in 1921.

[3] Atkins ('22) reports the salt error to be almost double that found here.

finding temperatures from some depth below the surface. All determinations were ordinarily made in duplicate and, oxygen excepted, frequently in triplicate.

TABLE I.

SHOWING COMPARATIVE RESULTS OF USING THREE INDICATORS IN
DETERMINING THE pH OF SEA WATER.

Indicator.	I.	II.	III.	IV.	V.
Dibromthymolsulphonephthalein......	8.0 +	8.0 +	8.0 +	7.3	8.0 +
Thymolsulphonephthalein...........	8.0 +	8.0 +	8.2	8.0 −	8.2
Phenolsulphonephthalein.	8.0 +	8.0 +	8.2	7.3	8.2

III. SERIES OF ASSOCIATIONS.

The animal associations of the Woods Hole littoral can be divided into two series either on the basis of the character of the bottom, or of physiographic action. On either basis the resulting series are the same, for the eroding shores of the region are all rocky and the depositing shores have a shifting bottom composed of mud or sand. Within these series the associations may readily be arranged in the order of their physiographic age.

The associations of the rocky eroding shores as studied are: (1) open water; (2) wharf pilings; (3) exposed rocks; (4) protected rocks. The associations of the depositing shores likewise begin with (1) the open water and typically continue as follows: (2) those of the sand bar; (3) with the deposition of mud this becomes the muddy sand association in which eel grass begins; (4) with further deposition of mud and as the eel grass grows longer one finds the *Cumingia* association and in deeper water where still more muck has been deposited, the *Scoloplos acutus* association. These make up the two main associations of the eel grass and muck, which extend to the margin of the eel grass in about a foot of water at low tide. Between this level and the shore at about low tide lies the marginal muck association (5) which gradually gives way to the intertidal associations, the lower of which may be called the *Mya* association and occupies the region from just below low tide to about the half tide mark. The upper association is marked by the abundance of the snail *Melampus* and may well be called by that name. In places the *Ucas* are more abundant, but such localities have not been studied in this report.

In order to understand this typical arrangement of associations in the depositing shore series it is necessary to know that in the flats of the region there is usually a sand bar formed at the outer edge of the flat which is frequently uncovered at low tide; farther back on the flats the water becomes slightly deeper in the region of mud and sand and then decidedly deeper until one reaches the *Scoloplos acutus* association of the eel grass and muck region. As one nears the shore the water becomes shallow again.

While the series as given is typical of the region there are several other possibilities which will be discussed in their proper places.

1. *The Rocky Eroding Shore Associations.*

a. The Open Water Association.—The results of analyses of water samples from four inshore stations are shown in Table II.

TABLE II.

SHOWING SALINITY, TEMPERATURE, OXYGEN CONTENT AND pH OF
OPEN WATER FROM INSHORE STATIONS.

Date.	Time.	Depth Ft.	Tide.	Temp. ° C.	pH.	O. in c.c. per L.	Salinity %.	Light.	Coll. No.
1920 8/16	10:50	14	High	21	I. 8.0	—	—	Dull	11
	11:10	22	High	21	8.0	3.93	—	Dull	12
9/3	9:15	22	Do.	20	8.0 −	4.37	3.02 3.05	Bright	107[1]
8/16	4:00	15	Sound to Bay	22	II. 8.0 +	—	—	Dull	22
	4:10	10	Do.	22	8.0	—	—	Dull	23
1921 9/12	11:45	12	Do.	21	8.0	5.43	3.14	Dull	122
					8.0	5.38	3.14		
				21	8.0	5.38	3.14		
					8.0	5.38	3.14		
				21	8.0	5.35	3.14		
				21	8.0	5.38	3.14		
				21	8.0	5.35	3.13[2]		
					8.0	5.33	3.12[2]		
					8.0	5.38	3.12[2]		
					8.0	5.38	3.12[2]		
1920 9/3	5:00	12	Bay to Sound	21	III. 8.1	—	3.04	Bright	120
8/31	4:10	24	Low	22	IV. 8.2	6.14	—	Rainy	100
	4:10	24	Low	22	8.0	5.84	3.09	Rainy	100′

[1] Collection 7 feet down.
[2] Determination in laboratory.
[3] Collection made 12 feet down.

Part I. is from collections made off the Bay opening of Northwest Gutter about 75 yards directly west of the corner of the channel made by the shoulder of Uncatena Island. Part II. is from the passage in Woods Hole and the third part is from the opening of Lackey's Bay into Vineyard Sound. The latter is influenced by the current carrying water from the extensive submerged vegetation of this region. Part IV. is from the mid-mouth of Gansett Bay. The surface water here also shows the effect of proximity to vegetation. The depths are those given in the U. S. Coast and Geodetic Survey charts. The last two divisions are given for comparison only and will not be considered in discussing open water conditions since they are obviously atypical.

The invertebrate animals to be found near the surface in such locations are largely plankton. A typical collection taken with miller's bolting cloth net from a Bay tide in Woods Hole at 10:00 A.M., August 8, 1917, is shown in List 1. The collection was studied the afternoon after it was taken by the class in Invertebrate Zoölogy of the Marine Biological Laboratory with the assistance of seven instructors. The list is based on the findings of approximately sixty people in a two hours search.

LIST 1. SHOWING THE MORE CONSPICUOUS ANIMALS TAKEN IN ONE TOWING IN THE HOLE.

Animals.	Number Found.	Animals.	Number Found.
PROTOZOA		BRYOZOA	
Ceratium	8	*Cyphonautes*	5
CŒLENTERATA		CRUSTACEA	
Actinula of *Tubularia*	2	*Nauplius*	3
Hydromedusæ	12	*Zœa*	5
NEMERTINI		*Mysis*	4
Pilidium of *Cerebratulus*	1	*Megalops*	5
CHÆTOGNATHA		Copepoda	∝
Sagitta	2	*Cypris* larvæ of *Balanus*	1
ECHINODERMA		MOLLUSCA	
Bipinnaria	12	Lamellibranch veligers	4
Brachiolaria	5	Gasteropod veliger	∝
Pluteus	2	CHORDATA	
ANNELIDA		*Appendicularia*	14
Dinophilus	5	*Botryllus* tadpoles	1
Trochophores	2		

b. Wharf Piling Association.—The animals in this association, whether free living, attached, or living among attached forms, are more abundant at the outer end of the piers where the water

is deeper and the wave action is less violent. The conditions in the water, so far as investigated, are given in Table III. It will

TABLE III.

Date 1920.	Time.	Depth in Ft.		Temp.	pH.	O_2 in c.c. per L.	Salinity.	Collection Number.
		Water.	Collection.					
8/21	8:30	12 +	12	19	8.0	4.48	—	40
	8:45	12 +	1	19	8.0	—	—	40
	9:20	10	10	20	8.0	—	2.97	41
	9:40	1	1	20	8.0	—	—	42
	10:10	12 +	0.25	—	—	6.0	—	43
8/22	9:40	12 +	1	20	—	4.36	—	44
	9:40	12 +	1	20	—	4.26	—	45
	10:00	12 +	12	20	—	4.16	—	46
	10:00	12 +	12	20	—	4.26	—	47
	10:35	12 +	1	—	—	4.11	—	48
	10:35	12 +	12	—	—	4.06	—	49
8/28	3:30	1	1	21	8.0	5.25	3.01	85
	3:45	12 +	12	20	8.0	5.25 5.15	3.07	86
	4:00	12 +	1	21	8.0	5.25	3.13	87

be noted that the pH was constant in all locations; that the oxygen content was relatively constant for each location, with the exception of one collection taken at Crane's Wharf just after the passing of a Nantucket steamer which caused a decided increase. There is no evidence of vertical gradients among the factors analyzed to explain the vertical distribution of animals found on the wharf pilings; nor is there an indication of horizontal gradients along the wharf save in salinity.

All the animals found in collecting on Crane's Wharf pilings are given in List 2. The figures give an arbitrary means of showing relative abundance and are based on the number of collecting teams reporting each animal in one afternoon's work early in July, 1920. Absence of such figures shows that the animals were not found at that time.

[1] Low tide and bright sunlight throughout.

LIST 2. SHOWING THE INVERTEBRATE ANIMALS TAKEN FROM CRANE'S WHARF PILES IN NINE YEARS' COLLECTING.

The figures give an indication of comparative abundance in 1920 and are based on the number of collecting teams reporting the several species. Absence of figure indicates that the animal was not taken during this trip.

	Sessile	Free
PORIFERA		
Chalina arbuscula	7	
Cliona celata(?)	7	
Grantia ciliata	7	
Leucosolenia botryoides	5	
Microcione prolifera	7	
CŒLENTERATA		
Campanularia sp	1	
Clytia bicophora	7	
Eudendrium album		
Eudendrium ramosum		
Gemmaria gemmosa	1	
Hydractinia echinata		
Metridium dianthus	6	
Obelia commissuralis	4	
Obelia geniculata		
Pennaria tiarella	1	
Sagartia luciæ	1	
Sagartia leucolena	1	
Schizotricha tenella		
Sertularia pumila		
Tubularia crocea	7	
PLATYHELMINTHES		
Tetrastemma vermiculum	1	
Lineus bicolor		
NEMATHELMINTHES		
Pontonema marinum	5	
ECHINODERMA		
Arbacia punctulata		3
Asterias forbesi		5
Asterias vulgaris		1
Henricia sanguinolenta		
ANNELIDA		
Arabella opalina		1
Amphitrite attenuata		2
Autolytus cornutus		1
Harmothœ imbricata		5
Hydroides hexagonus	5	
Lepidonotus squamatus		7
Lepræa rubra		
Nereis pelagica		7
Nicolea simplex		2
Podarka obscura		2
Polycirrus eximeus		3
Polydora sp		
Phyllodoce catenula		
Sabellaria vulgaris		
Spirorbis spirorbis	6	
Sthenelais leidyi		
Thelepus sp		
BRYOZOA		
Ætea anguina	5	
Bugula turrita	7	
Bicellaria ciliata		
Crisia eburnea	7	

	Sessile	Free
Lepralia pertusa		
Membranipora pilosa	6	
Flustrella hispida	2	
Schizoporella unicornis	6	
ARTHROPODA		
Amphithœ rubricata		2
Autonœ smithi		1
Anoplodactylus lentus		2
Balanus balanoides		7
Balanus eburneus		2
Caprella geometrica		7
Erichsonella filiformis		1
Idothea baltica		2
Libinia dubia		6
Libinia emarginata		6
Lygidia oceanica		2
Orchestia agilis		
Palæmonetes vulgaris		
Palene empusa		1
Pinnotheres maculata		2
Panopeus sayi		6
Talorchestia longicornis		
Tanystylum orbiculare		2
MOLLUSCA		
Acmea testudinalis		1
Anomia aculeata	1	
Anomia ephippium	4	
Arca pexata		
Bittium alternatum		4
Busycon carica		
Columbella avara		6
Columbella lunata		7
Crepidula convexa		
Crepidula fornicata		3
Crepidula plana		3
Coryphella gymnota		5
Lacuna vincta		4
Littorina litorea		7
Littorina palleata		1
Littorina rudis		1
Modiolus modiolus	1	
Mytilus edulis	6	
Teredo navalis		3
Odostomia sp		
Urosalpinx cinereus		7
CHORDATA		
Amaræcium constellatum	6	
Botryllus schlosseri		
Didemnum lutarium	7	
Molgula arenaria	1	
Molgula manhattensis	4	
Molgula papillosa	1	
Perophora viridis	6	
Styela partita	7	

Table IV. shows data taken from the records from the U. S.

TABLE IV.

SHOWING TEMPERATURE AND SALINITY FROM THE NOON READINGS
OF THE U. S. FISH COMMISSION AT THEIR WOODS HOLE
WHARF FOR AUGUST, 1920.

The raw data in sp. gr. and Fahrenheit have been converted to percentage of
salinity and the centigrade scale for purposes of comparison.

Day.	Salinity.	Temperature.	Day.	Salinity.	Temperature.
1	3.20	21.7	16	3.22	22.2
2	3.19	20.8	17	3.30	22.5
3	3.205	21.1	18	3.285	21.9
4	3.18	20.6	19	3.19	20.8
5	3.215	20.0	20	3.185	20.0
6	3.24	20.3	21	3.20	20.6
7	3.25	20.3	22	3.215	20.1
8	3.24	20.6	23	3.235	20.6
9	3.235	21.9	24	3.235	20.6
10	3.24	22.2	25	3.215	21.1
11	3.22	21.7	26	3.21	20.8
12	3.24	22.2	27	3.205	21.1
13	3.24	22.2	28	3.21	21.4
14	3.24	22.2	29	3.21	21.4
15	3.21	21.7	30	3.04	21.7
			31	3.065	21.7

Fish Commission from their collections off the Fish Commission
Pier and is given for comparison with records from wharf pilings
and also with those from open water.

The vertical distribution in the wharf piling association is
marked. Near the high tide level may be found the *Littorinas*,
Urosalpinx, *Balanus balanoides* and an occasional *Asterias forbesi*.
Nearer the water at low tide are *Mytilus* clusters and *Modiolus
modiolus* sheltering *Libinia dubia* and *Lepidonotus*. Below these,
normally under water at all times, come *Amarœcium* and *Styela*
and other pile dwelling chordates. Here are found the sponges,
Microcione, *Grantia* and *Cliona* and the hydroids such as *Tubu-
laria* and *Schizotricha*. With all these there is the attendant
fauna of worms, *Lepidonotus*, *Harmothoe*, *Sthenelais*, *Nereis
pelagica* and *limbata;* the sea slug *Coryphella* and the *Columbella*
snails are also abundant. Here also are *Bugula* and the en-
crusting bryozoans, *Schizoporella*, *Lepralia*, *Membranipora* and
the like. Below these are the sea anemones, *Metridium*.

The animals mentioned may be displaced somewhat from the order given but as one looks down the outer piles at Vineyard Haven four distinct bands can easily be recognized: The *Balanus* band well above the low water level; the *Mytilus* band near low water, the *Amarœcium-Tubularia* band which shows as a more brilliantly colored region and finally the dull brown of the *Metridium* band which extends well down toward the bottom of the pilings.

Apart from the obvious relations in the upper portion where independence of water is the controlling factor, the vertical distribution may be in part the effect of light differences particularly on the free swimming larvæ. Grave ('20) has found that *Amarœcium* tadpoles, for example, are first positive but soon become negative to light. In part the position may be a response to gravity for the same tadpoles are at first negative in their reaction to gravity and later become positive. When both light and gravity act as they would in nature, the tendency of the tadpoles to settle to the bottom of the experimental cylinder was most marked and occurred in 85 per cent. of the cases. The banding may also be controlled in part by reactions to pressure and to conditions in the water that could not be detected by the measurements made. On the whole Grave is inclined to attribute the distribution of *Amarœcium* colonies to the effect of gravity during the active, free-swimming life of the tadpoles.

c. Rock Associations.—There are two distinct types of animal associations to be found in the region dominated by rocks. First, there are the animals and plants attached to the rocks and rockweeds or living among those so attached and, second, there are the animals living in the substratum under and sometimes between the rocks. These last are closely similar to the animals of the mud or sand flats near which the rocks are located. No attempt has been made to get water samples exactly characteristic of the second community, since in this work the main attention was focused on the animals of the rocks.

Two animal associations of the rocks proper are easily recognized.. On the exposed rocks, where there is little rockweed, the animals are mainly found under the edges of the rocks out of the direct sweep of the waves. In protected regions, the rocks are covered with the rockweeds, *Ascophyllum* and *Fucus*. Here

many of the animals are to be found on the upper surface of the rocks under the protecting rockweed. This association may be designated as the protected rock association or more obviously as the rockweed association.

(1) *Exposed Rock Association.*—The association inhabiting the exposed rocks was much studied at the opening of Northwest Gutter into Buzzards Bay. The rocks are exposed to the full sweep of the waves across the Bay and support a sparse growth of *Ascophyllum* and *Fucus*.

Similar conditions are common along the coast. They were studied in three localities: (1) At the outer point of East Buck Island in Lackey's Bay; this differs only in that submerged vegetation lies closer to the rocks on the shore side and extends around the rocks on the Sound side. The waves from the Sound break freely over these rocks. (2) At Gansett on the point which separates Gansett Bay from Quissett Harbor. The whole bottom nearby is covered with eel-grass while in the immediate vicinity *Sargassum filipendula*, *Ascophyllum* and *Fucus* grow in abundance. These rocks are frequently reached by the direct waves from Buzzards Bay and are themselves practically free of rockweeds. (3) In the region of Southwest Gutter where the rocks are protected from the sweep of the waves but are washed by strong and almost constant tidal currents coming now from the Sound and now from the Bay. The direction of the current makes little difference in the character of the water since in either case it passes through tortuous, shallow passages supporting a large amount of submerged vegetation. While there are many plants nearby, these rocks do not support a large amount of plant life. Water analyses from this association are given in Table V.

The animals most common in the exposed rock association are the sponges, *Microcione* and *Cliona;* the actinians, *Sagartia leucolena* and *luciæ* and *Metridium;* the starfish, *Asterias;* the annelids, *Harmothoe*, *Hydroides*, *Lepræa* and *Lepidonotus;* the bryozoan *Bugula* and various incrusting forms; *Balanus balanoides* and *eburneus*, the barnacles; *Chætopleura*, the chiton; the bivalve molluscs, *Anomia* and *Mytilus;* the snails, *Acmea*, *Littorina litorea*, *Eupleura*, *Purpura* and *Urosalpinx;* and the chordates, *Amarœcium*, which is usually and characteristically

TABLE V.

SHOWING OXYGEN CONTENT, pH, TEMPERATURE AND SALINITY OF WATER FROM THE ANIMAL ASSOCIATION OF THE EXPOSED ROCKS.

Date 1920.	Depth Water.	Tide.	Temp.	pH Bot.	pH Sur.	O₂ Bot.	O₂ Sur.	Time.	Light.	Salinity.	Coll. No.	Place.
8.14	2 ft.	Flowing	21	8.1	—	—	—	4:30	Dull	3.04	9	Gutter (N.W.)
8.18	1 ft.	Ebbing almost low	21	8.1	—	3.62	—	3:50	Dull	—	37	
8.23	1 ft.	Low	20	8.0	—	4.41	—	9:00	Dull	3.04	50	
8.24	3 ft.	Flowing almost low	20	8.0	—	4.95	—	11:10	Bright	2.94	65	
9.3	7 ft.	2/3 in	20	8.0	—	4.25	—	10:00	Bright	2.99	108	
8.25	2 ft.	Low to bay	21 on surface	—	8.2	—	5.45	11:35	Bright	—	79	Gutter (S.W.)
8.25	6 ft.	Low to bay	21 on surface	—	8.2	—	6.54	12:45	Bright	3.01	81	
9.3	6 ft.	Slack	21 S.	—	8.2	—	5.39	2:30	Bright	3.01	116	Lackey's Bay
8.25	1.5 ft.	Low	22 S.	8.2	—	7.03	—	1:30	Bright	3.04	82	
9.3	1 ft.	Ebbing	21	8.2	—	4.16	—	4:40	Bright	3.04	120	Gansett
8.30	2 ft.	Low	23	8.3	8.3	8.56	—	2:45	Bright	—	89	
8.30	2.5 ft.	Flowing	22	8.3	—	8.41	—	3:45	Bright	—	91	
8.30	3 ft.	Flowing	—	—	8.3	—	—	4:10	Bright	—	93	
9.1	6 ft.	Near high	22	8.0	8 0	5.15	—	11:30	Overcast	3.05	96	
9.1	2 ft.	Low	21	8.2	—	6.14	—	3:45	Overcast	3.02	104	

LIST 3. SHOWING ANIMALS COMMONLY FOUND IN THE EXPOSED
ROCK ASSOCIATION.

Animals starred may be taken above low tide mark.

PORIFERA
Cliona celata(?)
Grantia ciliata
Leucosolena botryoides
Microcione prolifera*

CŒLENTERATA
Astrangia danæ
Clava leptostyla
Hydractinia echinata*
Metridium dianthus
Sargartia leucolena
" luciæ*
" modesta
Sertularia pumila

PLATYHELMINTHES
Stylochus ellipticus

ECHINODERMA
Arbacia punctulata
Asterias forbesi*

ANNELIDA
Harmothoe imbricata*
Hydroides hexagonus*
Lepræa rubra
Lepidonotus squamatus*
Nicolea simplex
Polycirrus eximeus
Spirorbis spirorbis*
Sabellaria vulgaris*

BRYOZOA
Alcyonidium sp.
Bugula turrita*
Crisia eburnea*
Flustrella hispida
Lepralia pertusa
Membranipora pilosa*
Schizoporella unicornis*

CRUSTACEA
Amphithoe

Balanus balanoides*
Balanus eburneus
Cancer irroratus*
Idothea metallica
Idothea baltica*
Pagurus longicarpus*
Panopeus sayi*
Sphæroma quadridentatum*
(Under rocks)

MOLLUSCA
Chætopleura apiculata
Anomia ephippium*
aculeata
Modiolus modiolus*
Mytilus edulis*
Acmea testudinalis
Bittium alternatum
Cerithiopsis emersonii
Columbella avara
lunata*
Coryphella gymnota
Crepidula fornicata
convexa
plana*

Eupleura caudata

Lacuna vincta*
Littorina litorea*
palliata
rudis*
Purpura lapillus*
Odostomia sp.
Urosalpinx cinereus*

CHORDATA
Amaræcium constellatum*
Botryllus schlosseri* .
Didemnum lutarium
Molgula manhattensis
Perophora viridis
Styela partita

present in small clusters, and *Styela*. A more complete list is given in List 3, which while not exhaustive, does represent the association in an adequate manner. The species starred were taken above low spring tide in collecting on the rocks at Kettle Cove, August 4, 1921.

The chance of differentiation in the vertical distribution is not so great here as on the wharf pilings and, in addition, the spaces between the rocks and under them hold moisture better than in the case of the pilings so that the break at the tide level is less marked. Nevertheless the animals to be found on the rocks vary greatly with the height above mean low tide. Differences found in animal life and in water analyses at different tide levels are given in List 4 and Table VI. The high tide levels were studied on the Northwest Gutter rocks. The tidepool and region below low tide are from data obtained at Gansett. There is no reason to suppose that high tide conditions are different in the two places.

The tidepool was well separated from the water of the Bay. It contained about 6–12 inches of water and was one foot wide by three feet long. Some rockweeds were present but not in the numbers common in a typical rockweed association.

It is possible that the greater acidity combined with the decreased oxygen content of the water at the margin at high tide might cause sensitive larvæ to turn back. However when the waves are running well they would have no power in the matter. It is also possible that the sensitive animals of the exposed rock association are kept out of the tide pools by the great range in acidity which was over twice that found on the nearby rocks below low tide and, as seen in List 4, the animals present are fewer in numbers and in species.

(2) *Protected Rocks or Rockweed Association.*—The best example of the rockweed association to be studied was found in the creek between Southwest Gutter and Hadley Harbor. In this place the rockweeds, *Ascophyllum* and *Fucus*, form a patch about two rods square which reaches half way across the creek. The water ranges from six inches to four feet in depth. Between the rocks on the bottom are small patches of gravelly sand mixed with humus. Large boulders, which extend well out of the water at

TABLE VI.

Showing Oxygen Content, pH, Temperature and Salinity of Water over Different Tidal Levels of More Exposed Rocks.

Date 1920.	Depth of Sample.	Tide.	Temp.	pH. Bottom.	pH. Surface.	O_2 Bot.	Light.	Time.	Salinity.	Coll. No.	Place.
8/16	2 ft.	High	21	7.8	—	3.56	Dull	9:10	—	10	Gutter (N.W.)
8/18	1 ft.	Half	22	7.9	—	3.72	Rainy	9:30	—	24	
9/30	.7–1 ft.	Low	24	7.9	—	3.18	Bright	3:00	—	90	Tide pool Gansett
8/30	1.25 ft.	Flowing	24	8.4	—	8.32	Bright	4:00	—	92	
8/30	1.5 ft.	Flowing	23	8.4	—	8.02	Sunny	4:20	—	94	
9/1	3 ft.	Ebbing	22	7.7	—	5.15	Overcast	11:40	3.05	97	
9/1	.7 ft.	Flowing	22	8.0	—	5.94	Sunny	4:00	3.06	105	
8/30	2 ft.	Low	23	8.3	8.3	8.56	Bright	2:45	—	89	Below low tide Gansett
8/30	2.5 ft.	Flowing	22	8.3	—	8.41	Bright	3:45	—	91	
8/30	3 ft.	Flowing	—	—	8.3	—	Bright	4:10	—	93	
9/1	6 ft.	Near high	22	8.0	8.0	5.15	Overcast	11:30	3.05	96	
9/1	2 ft.	Low	21	8.2	—	6.14	Overcast	3:45	3.02	104	

List 4. Showing Animals Found at Different Tide Levels on More Exposed Rocks.

A. At extreme high tide; NW. Gutter rocks.
  Orchestia agilis.
  Talorchestia longicornis.
B. At Half Tide; Same Place.

CŒLENTERATA:
  Sertularia pumila, much.
CRUSTACEA
  Balanus balanoides, very abundant.

MOLLUSCA:
  Mytilus edulis, several, small.
  Littorina litorea, very many.
    rudis, several.
    palliata, some on
      Ascophyllum.

C. Tide Pool among Rocks, Gansett.

PORIFERA
  Cliona celata. (?)
  Microcione prolifera, some.

D. Below Low Tide, Gansett.

Ten times as much as in *C.*
Much, more than in *C.*
Leucosolenia botryoides, sev. colonies.
Grantia ciliata.

CŒLENTERATA:
  Sagartia leucolena, few

Several
Astrangia danæ, sev. colonies
Metridium dianthus, several.
Sertularia pumila.

BRYOZOA:
  Bugula turrita

More here.
Crissia eburnia, 1 colony.

ANNELIDA:
  Harmothoe imbricata, several.
  Hydroides hexagonus
  Lepræa rubra, few.
  Lepidonotus squamatus
  Spirorbis spirorbis.

Several, more than in *C.*
Many more than in *C.*
Many.
About same as *C.*

CRUSTACEA:
  Balanus balanoides, very many

Some
Gammarus sp. many.

MOLLUSCA:
  Anomia ephippium, several small.
  Acmea testudinalis
  Cerithiopsis emersonii
  Littorina litorea, very abundant.
  Urosalpinx cinereus

Large ones here.
More than in *C.*
None.
Fewer than in *C.*
Many more than in *C.*
Chætopleura apiculata, few large.
Anomia aculeata, several small.
Modiolus modiolus, few.
Crepidula plana, few.
Littorina rudis, few.

CHORDATA:
  Stylela partita

Few in both.
Amarœcium constellatum, much.

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low tide, support masses of the rockweeds. The water here is constantly changed by the strong tidal current.

A similar situation, except for the tidal current, was examined at the inshore end of Gansett Bay. The region there is in the form of a triangle with the shore for the long base and with the apex made by a large boulder lying about two rods off shore at low tide.

A partial list of the animals to be found in this association, divided according to strata, is given in list 5. As might be expected, animals usually found only at or near low tide, occur here well above low tide in some abundance on account of the protection furnished and the water held by the rockweeds.

LIST 5. SHOWING REPRESENTATIVE ANIMALS OF THE ROCKWEED
ASSOCIATION BY STRATA.

A. Animals on or Among the Rockweed when Left Exposed by the Tide.

CŒLENTERATA:
  Clava leptostyla,
  Obelia geniculata,
  Sertularia pumila,
  Sagartia luciæ.

ECHINODERMA:
  Asterias forbesi.

BRYOZOA:
  Alconidium sp.?,
  Bugula turrita,
  Flustrella hispida.

ARTHROPODA:
  Amphithoe rubricata,
  Caprella geometrica,
  Balanus balanoides,
  Anoplodactylus lentus,
  Limulus polyphemus.

MOLLUSCA:
  Mytilus edulis,
  Littorina litorea,
  Littorina palliata,
  Columbella avara,
  Columbella lunata.

B. Animals from the Rocks Below the Rockweed Stratum from First Six Rocks Examined.

PORIFERA:
  Cliona celata, (?)
  Microcione prolifera,

CŒLENTERATA:
  Sagartia leucolena.

ECHINODERMA:
  Asterias forbesi.

BRYOZOA:
  Lepralia pertusa,
  Membranipora pilosa,
  Schizoporella. unicornis.

ANNELIDA:
  Harmothoe imbricata,
  Lepidonotus squamatus,
  Hydroides hexagonus,
  Polycirrus eximeus.

ARTHROPODA:
  Cancer irroratus.

MOLLUSCA:
  Mytilus edylis.

CHORDATA:
  Styela partita.

C. Animals Found in Brief Examination on Surface of Substratum.

CRUSTACEA:

    Cancer irroratus,

    Libinia dubia carrying the sponge, *Cliona celata,* (?)

    Pagurus longicarpus carrying the hydroid, *Hydractinia echinata,*

    Panopeus sayi.

GASTEROPODA:

    Littorina litorea, very numerous.

D. Animals Found in Sampling the Substratum. (*Three spadesful were dug.*)

PLATYHELMINTHES:	ANNELIDA:
Lineus ochræus.	*Clymenella torquata,*
	Lepræa rubra,
MOLLUSCA:	*Lumbrinereis tenuis,*
Cumingia tellinoides,	*Nereis limbata,*
Nassa trivitatta.	*Pectinaria gouldii.*

Water analyses for this association are shown in Table VII. The table includes the last six items from the preceding table, since the conditions described in those collections are as characteristic of the one as of the other. A discussion of the water analyses for this and the other associations of the eroding shore series is reserved to a later section.

Here this series logically ends so long as the shores remain under erosion with a gradual wearing away of the land to expose more rocks, which would result in the association of the exposed rocks moving gradually forward as erosion proceeds. In case the shore line should remain stationary for sufficient time, the rocks might be worn away to sand and this association might conceivably pass gradually into that of the sand bar which will be considered in the next section. On the other hand if the configuration of the land changes so that currents no longer cause erosion, but deposition sets in, then in the region of the present rock associations one may find sand deposited and the sand bar association might be reached by that route. If the region becomes still more quiet, mud might be deposited and the rock association would then pass to the *Mya* association and with further deposition, pass into the *Uca* or *Melampus* stage. The physical factors correlated with these associations will be given on a later page and anyone interested in completing this series on either of these possibilities, can readily do so.

TABLE VII.

SHOWING OXYGEN CONTENT, TEMPERATURE, pH AND SALINITY OF THE ROCKWEED ASSOCIATION.

Date 1920.	Depth of Sample.	Tide.	Temp.	pH. Bot.	pH. Sur.	O₂ Bot.	O₂ Sur.	Time.	Light.	Salinity.	Coll. No.
						Gansett.					
8/30	2 ft.	Low	23	8.3	8.3	8.56	—	2:45	Bright	—	89
8/30	2.5 ft.	Flowing	22	8.3	—	8.41	—	3:45	Bright	—	91
8/30	3 ft.	Flowing	—	—	8.3	—	—	4:10	Bright	—	93
9/1	6 ft.	Near high	22	8.0	8.0	5.15	—	11:30	Overcast	3.05	96
9/1	1 ft.	Near low	23	8.0	—	5.35	—	2:45	Bright	2.97	103
9/1	2 ft.	Low	21	8.2	—	6.14	—	3:45	Overcast	3.02	104
						S. W. Gutter Creek.					
9/3	2–2.5 ft.	Slack	19	8.0	—	4.75	4.75	2:15	Bright	3.01	115
8/25	1–1.5 ft.	Low to Bay	21	8.0+	8.0+	4.75	5.35	9:10	Bright	2.98	77

2. *Associations of Depositing Shores.*

Notwithstanding the slight amplitude of tides in the Woods Hole region, there are a number of fairly extensive tidal flats which furnish the basis for the associations of the depositing shores. The best of these near the Laboratory is the flat opening off Northwest Gutter just before it enters Buzzards Bay. The majority of collections to be reported were made at this place. Verification studies were carried on in Lackey's Bay, at Gansett, and in Southwest Gutter Creek.

The different animal habitats to be found on the flats grade slowly from one to another so that it becomes impossible to get hard and fast limiting lines. This is true even of the more extensive flats, those at North Falmouth for example, but it is particularly marked in regions such as Gansett where the water deepens fairly rapidly, leaving only narrow zones along the margin. Similar jumbling of habitats is to be found in the salt water creeks of Hadley Harbor and is more characteristic of the Woods Hole coasts than are the more gradual transitions found on the flats proper.

Theoretically the youngest stage in this series is that shown in (*a*) the association of the open water. The conditions in such positions have already been discussed so we shall proceed immediately to the associations of the flats.

b. The Sand Bar Association.—In this region tidal flats are typically separated from the open water by sand bars which may be much exposed at low spring tides, or just covered at low neap tides. The outer part of the bar is relatively pure sand which gradually becomes mixed with humus as one moves back on the flats. The characteristic animals of such an association are given in list 6.

In places practically pure cultures of *Scoloplos fragilis* occur in the sand and if an animal name for the association is desired it might well be called the *Scoloplos fragilis* association. •

Where the sand bar extends out to the margin one finds the expected evidences of vertical distribution depending on tide level. At the upper tidal limit congregating under the eel grass occur great numbers of the beach flea, *Orchestia agilis;* burrowing in the sand are the *Talorchestia longicornis* and locally, *Hippa talpoida.*

CŒLENTERATA:
  Hydractinia echinata on shells oc-
    upied by *P. longicarpus.*

NEMERTINI:
  Cerebratulus lacteus,
  Micruri leidyi.

ECHINODERMA:
  Leptosynapta inhærans.

ANNELIDA:
  Clymenella torquata,
  Diopatra cuprea,
  Phyllodoce sp.,
  Scoloplos fragilis,
  Scoloplos robustus.

ARTHROPODA:
  Crangon vulgaris,
  Ovalipes ocellatus,
  Pagurus longicarpus,
  Limulus polyphemus.

MOLLUSCA:
  Crepidula convexa, on hermit crab
    shells,
  Crepidula plana, on hermit crab
    shells,
  Ensis directus,
  Nassa trivittata,
  Natica duplicata, with egg collars.

CHORDATA:
  Dolichoglossus kowalevskyi.

The conditions of the water over this association are shown in Table VIII. The last item in the table was from Blind Gutter

TABLE VIII.

SHOWING TEMPERATURE, SALINITY, OXYGEN CONTENT AND pH OF SEA
WATER OVER THE SAND BAR ASSOCIATION.

Date 1920.	Time.	Depth Water in Ft.	Tide.	Temp. ° C.	pH.	O₂ in c.c. per L.	Light.	Salin-ity.	Collec-tion Num-ber.
8/14	3:15	1.0	Low	24.5	8.25	—	Dull	3.10	4
8/16	11:30	6.0	High	22.0	8.0	—	Dull	—	13
8/18	2:20	1.5	Ebbing	22.0	8.1	—	Rainy	—	30
	4:00	0.5	Low	21.0	8.1	—	Rainy	—	38
8/23	9:35	0.75	Low	20.0	7.6	3.91	Dull	—	51
8/24	11:15	0.5	Flowing	20.0	8.0	4.63	Bright	2.99	66
9/3	10:30	5.0	Flowing	20.0	8.0	3.95	Sunny	2.99	109
9/3	4:30	1.25	Ebbing	20.0	8.2	4.16	Sunny	3.02	119[1]

Bar. The others are from Northwest Gutter Bar. With the exception of one reading, made in triplicate, the pH ranges from 8.0 to 8.25. In this respect it approaches open water. The observed range is greater than that of the nearby rocks. The salinity is similar to that of the rocks and less than that of the open water. The oxygen content resembles that of exposed rocks where rockweeds are absent. The temperature range is greater than in any other association so far studied and the

[1] Blind Gutter Bar.

temperature at the upper surface of the sand when fully exposed to the afternoon sun must run much higher than the recorded 24.5° C. observed for the water under these conditions. The pH at Blind Gutter shows the effect of the neighboring vegetation which lies on both sides of this bar.

c. *Muddy Sand Association.*—The association of muddy sand shades insensibly into the preceding animal community on its outer side. On its inner side it includes the short eel grass (*Zosteria marina*) which extends well out on the sand and then gradually passes into the eel grass and muck association. The most characteristic animal of the association is the sipunculid worm, *Phascolosoma gouldii* and the association might well be called the *Phascolosoma* association. Much of the muddy sand may be exposed at extreme low tide.

This community was most extensively studied at Northwest Gutter, but verification studies were run at other points. Common animals of the association are given in list 7 but the animals shown in the preceding list are also found here.

LIST 7. ANIMALS EASILY FOUND IN THE MUDDY SAND OR
Phascolosoma ASSOCIATION.

(See also List 6.)

CŒLENTERATA
  Hydractinia echinata,
  Sagartia luciæ.

ANNELIDA
  Arabella opalina,
  Clymenella torquata,
  Drilonereis longa,
  Glycera americana,
      dibranchiata,
  Lumbrinereis tenuis,
  Nereis limbata,
  Nereis virens,
  Pectinaria gouldii,
  Phascolosoma gouldii,
  Phyllodoce catenula.

MOLLUSCA
  Crepidula convexa,
  Lacuna vincta,
  Lævicardium mortoni
  Littorina litorea,
  Mya arenaria,
  Nassa obsoleta,
  Venus mercenaria.

ARTHROPODA
  Carcinides mænas,
  Heteromysis formosa.
  Libinia dubia,
  Limulus polyphemus,
  Palæmonetes vulgaris,
  Virbius zostericola.

CHORDATA
  Dolichoglossus.

The analyses of water over the muddy sand association (Table IX.) show an increase in range in all four factors considered when compared with the conditions found on the sand bar. The majority of these samples are from the edge of the short

eel grass. The effect of the eel grass on oxygen content and on pH is noticeable although not so extreme as will be found later. These effects are the expected result of photosynthesis.

TABLE IX.

SHOWING ANALYSES OF THE WATER TAKEN OVER THE MUDDY SAND OR *Phascolosoma* ASSOCIATION.

Date 1920.	Time.	Depth Water in Ft.	Tide.	Temp. °C.	pH.		O₂.		Salinity.	Light.	Coll.
					Bot.	Sur.	Bot.	Sur.			

Northwest Gutter.

8/14	2:10	.25 −.5	Low	25.5	8.45	—	10.39	—	3.10	Dull	1
	4:00	.08	Low	27.0	8.5	—	—	—	—	Dull	1′
	4:05	.5	Flowing	26.0	8.4	—	—	—	—	Dull	6
	4:30	.33	Flowing	27.0	8.45	—	—	—	—	Dull	8
8/16	3:55	1.5	Ebbing	22.0	8.1	—	5.30	—	—	Duller	15
8/18	10:05	4.0	Flowing	22.0	7.9+	8.0−	4.21	—	—	Rainy	25
	2:30	2.0	Ebbing	22.0	8.1	8.1	—	—	—	Rainy	31
	3:45	0.5	Ebbing	—	—	—	4.76	—	—	Rainy	36
8/23	10:00	1.0	Flowing	—	7.8	—	4.2	—	3.04	Dull	52
	1:15	3.0	Flowing	—	8.0−	8.0−	4.75	—	—	Sunny	58
8/24	2:15	2.5	Flowing	21.0	8.0+	8.0+	5.05	5.15	—	Sunny	73
9/3	12:50	5.0	High	21.0[1]	8.1	8.1	4.71	4.63	3.04[1]	Bright	113

Southwest Gutter Creek.

8/25	10:20	.08	Low	21	7.7	—	—	—	—	Bright	78
	10:20	.67	Low	21	8.0	—	5.25	—	2.97	Bright	78
	12:10	.75	Low	—	8.0	—	5.20	—	—	Bright	80

Gansett.

8/31	1:45	1.0	Ebbing	23	8.2	—	7.63	—	2.97	Sunny	98
9/1	1:15	3.0	Half	21[2]	8.2	8.0	7.08	6.19	2.96	Bright	101
	4:21	1.0	Flowing	23.5	8.4	—	8.61	—	—	Bright	106

d. Eel Grass and Muck Associations.—The associations of the eel grass and muck lie between the last discussed habitat and a region about a rod from the shore line of the type of flats under discussion. There at a depth of about a foot at low tide, the eel grass ends and the marginal muck association begins. Two major recognizable associations occupy this region: that characterized by the bivalve, *Cumingia tellinoides*, which may be

[1] Same at surface.

[2] 23 at surface.

called for convenience the *Cumingia* association and in deeper water and deeper muck, the région characterized by the annelid, *Scoloplos acutus*, which may give its name to this association.

On account of lack of time no water samples were taken over the *Cumingia grounds.* Enough collecting to yield 25 *Cumingia*, gave the following associates:

Annelida: *Polycirrus eximeus, Trophonia affinis, Amphitrite ornata, Glycera americana* and *Maldane elongata.*

Crustacea: *Panopeus sayi.*

Mollusca: *Tellina tenera, Solemia velum, Nucula proxima, Venus mercenaria, Lævicardium mortoni.*

Associated with these one usually finds the burrowing anemone, *Edwardsia elegans;* the swimming annelid, *Podarka obscura;* the shrimps: *Palæmonetes, Crangon,* and *Hippolyte;* and the green crab, *Carcinides.*

In both Northwest and Blind Gutters the *Scoloplos acutus* association occupies several acres.   At Gansett, the whole center of the bay supports a dense growth of eel grass and from general appearances, I should judge that it would belong to this association but it comes out into shallow water only in a relatively narrow zone and there some of the typical animals, *Thyone briareus*, for example, were not found.

LIST 8.   SHOWING THE COMMON ANIMALS FOUND ON, AMONG, AND AT THE ROOTS OF EEL GRASS IN THE CENTER OF NW. AND BLIND GUTTER FLATS.

A. ON EEL GRASS.

COELENTERATA:
 Sagartia luciæ (frequently many).
BRYOZOA:
 Bugula turrita (much).
 Schizoporella unicornis (some).
GASTEROPODA:
 Bittium alternatum (many).
 Crepidula convexa (several).

Crepidula fornicata (few).
Littorina rudis (few).
Littorina litorea (many).
Littorina palliata (few).
CHORDATA:
 Molgula manhattensis (very few).
 Botryllus schlosseri (little).

B. AMONG EEL GRASS.

ANNELIDA:
 Podarka obscura (frequently abundant).

CRUSTACEA:
 Virbius zostericola (very many).
MOLLUSCA:
 Pecten irradians (many).

C. IN MUCK AT ROOTS.

ECHINODERMA:
 Thyone briareus (frequently abundant).

ANNELIDA:
 Scoloplos acutus (many).

This association is in the region of tall eel grass which at high tide floats erect, allowing light to penetrate to the bottom. As the tide recedes, the grass falls over and finally at low tide forms dense mats which effectively shade the bottom except in queer open spaces where for some reason the eel grass fails to grow.

The eel grass supports a considerable fauna and flora. Davis (1911) lists 42 species of plants known to occur as epiphytes upon it in the more open situations. The number of animals found in the type of region under discussion is restricted. List 8 shows those found in Northwest and Blind Gutters in this association. The animals listed were collected in about three times the collecting time that gave the *Cumingia* list.

Above the mat of living eel grass at low tide, the temperature runs up as high as 32 degrees C. on a hot afternoon. Just below the matted eel grass, six inches from the water surface the temperature was 27, while at the bottom the thermometer registered 24 degrees. Thus there is a temperature gradient here of eight degrees in 30 inches.

Similar gradients of other environmental factors are present. Thus the oxygen content of the water at the bottom just above the *Thyone* and *S. acutus*, may fall as low as to give a mere trace with the Winkler method, when 24 inches higher, just below the eel grass mat, it was found to be 10.22 c.c. per liter and six inches higher, at the surface, 12.97 c.c. per liter. This makes a total gradient of 12.97 c.c. per liter in 30 inches. The pH gradient was found to be wholly similar: 7.3 at the bottom, 8.5, below and 9.0 above the eel grass matting.[1] These results are the natural concomitants of rapid photosynthesis at the surface of quiet water overlying thick muck.

The greater density was found at the bottom, but when the density was corrected for temperature the determinations showed a higher percentage of salinity at the surface. These and other details are shown in Table X. Particular attention is called to the wide range of variation in the different factors analyzed for this association. In the table, collections 99 and 102 are from

[1] Under laboratory conditions Atkins ('22) reports that sea water may come to have a ᴘH of 9.77 when jars containing *Ulva* are exposed to sunlight.

TABLE X.

SHOWING THE TEMPERATURE, OXYGEN CONTENT, SALINITY AND pH OF THE WATER OVER THE *Scoloplos acutus* ASSOCIATION.

Date 1920.	Time.	Depth in Ft.	Tide.	Temp. ° C.	pH. Bot.	pH. Sur.	O₂. Bot.	O₂. Sur.	Salin-ity.	Light.	Coll.
8/14	2:35	.25 -.5	Low	25.5	—	8.45	—	—	—	Dull	2
	3:00	.25 -.5	Low	25.0	8.25	—	—	—	—	Dull	3
	4:00	.25 -.5	Low	26.0	—	8.4	—	—	—	Dull	3'
	3:30	.33	Low	26.5	—	8.45	—	—	—	Dull	5
8/16	1:30	1.25	Ebbing	22.0	8.2	—	—	—	—	Duller	16
	2:10	1.0	Ebbing	23.0	7.9	—	—	—	—	Duller	18
	3:00	0.67	Ebbing	—	7.85	—	—	—	—	Duller	20
8/18	11:00	4.5	High	22.0	8.0	8.0	1.96	—	—	Rain	26
	2:45	1.6	Ebbing	—	8.0	8.2	—	—	—	Bright	32
8/23	10:25	1.0	Flowing	20.0	7.85	8.0	2.37	—	—	Dull	53
	1:30	3.0	Flowing	23.0	—	8.4	7.03	7.81	—	Sunny	58'
	2:00	3.0	Flowing	—	—	8.3	—	—	—	Sunny	59
8/24	9:55	0.75	Low	20.5	7.8 7.7	8.6− 8.6−	4.9	8.66	3.02	Sunny	63
	11:30	1.25	Flowing	24.0S	8.3	8.6−	5.69	9.9	—	Sunny	67
	1:30	2.0	Flowing	25.0S 24.0B	8.2	8.5	4.65	8.91'	—	Sunny	72
	2:20	2.5	Flowing	23.5S	8.3+	8.3	5.54	5.31	—	Sunny	74
	2:50	3.0	Flowing	26.0S	8.35	8.7	9.51	10.7	—	Sunny	75'
9/3	11:50	6.0	High	18.0B 20.0S	8.0	8.1	5.88	5.31	2.98B 3.01S	Bright	112
8/31	2:30	2.5	Ebbing	22.5B 26.0S	7.8	8.6	3.26	10.6	3.01	Sunny	99
9/1	2:00	3.0	Ebbing	22.0B 22.0S			2.28	7.68	2.99	Bright	102
8/25	2:10	2.5	Flowing	24.0B 32.0S	7.3	9.0	Tr.	12.97	2.88	Sunny	83
	3:05	2.5	Flowing	27.0M	8.5M		10.79M		—	Sunny	83'
9/3	3:40	2.5	Ebbing	20.0B 24.0S	7.8	9.0−	4.25	10.8	3.02B 3.11S	Bright	118
8/14	4:15	0.25	Low	26.0	8.0	—	—	—	—	Dull	7
8/18	2:00	3.5	Ebbing	22.0	7.7+	—	2.43	—	—	Dull	29
8/23	12:00	1.5	Flowing	20.0b	7.6	7.9	4.46	4.6	3.02	Bright	56
	1:00	2.1	Flowing	20.0	7.9	7.9	—	—	—	Bright	57
8/24	10:50	0.67	Low	22.0	8.1	8.1	4.95	—	—	Bright	64
9/3	1:00	4.5	High	21.0c	8.0	8.1	4.32	4.84	3.01c	Bright	114

Collections marked "S" are from the surface; "B," are from the bottom; 'M" are from six inches below the surface. Collections not otherwise labeled are from the bottom.

a. Collection No. 75, although taken after No. 74, was from further inshore and represents an earlier stage in the rising tide.

b. 21 at surface.

c. Same at surface.

Gansett; 83 and 118 are from Blind Gutter Flats. The others are from NW. Gutter Flats.

One open space in the eel grass, similar to those occurring scattered over the flats, except that it is larger and occurs near the margin, is to be found at NW. Gutter where a gravel spit extends from Uncatena Island partially across the creek leading to Hadley Harbor. On the Hadley Harbor side of the spit is a space covering some 2–3 square rods close up in the angle made by the gravel with the shore. The almost bottomless muck is covered over by a thick layer of old eel grass which acts as a very effective matting over the soft ooze below. Such eel grass is very resistant and decays slowly. The main tidal current flows past the edge of this eddy and thus prevents conditions from becoming as extreme as they would otherwise.

In addition to the numerous mud snails (*Nassa obsoleta*), one may find here: a few hermit crabs (*P. longicarpus*) carrying *Hydractinia* and the snail, *Crepidula convexa*. In places the common *Littorina litorea*, almost equals the mud snails in numbers and in other nearby regions only the latter are found. Some *Libinia dubia* are also on the surface while under the matting one finds nothing save a few *Scoloplos fragilis*.

The analytical data collected from this locality is shown in the latter part of Table X, commencing with collection No. 7. In many respects the conditions in this particular location resemble those of the *Thyone* association and seem to present a transition stage between the two associations. This will be discussed in detail in the last section.

e. Marginal Muck (Thyone) Association.—*Thyone* occurs in the preceding association as well as here, but since they are easily the most conspicuous animals in this environment and there are at least as many of them here as among the eel grass, and they may be absent in typical *S. acutus* conditions, the name is entirely appropriate.

The marginal muck association inhabits the region between the edge of the eel grass and low tide and overlaps both the preceding and the following associations. The eel grass on muddy shores usually begins to grow where the water is from one to two feet deep at low tide. This leaves a space from one to two rods wide on the inner side of Northwest and Blind Gutter Flats, bare of

vegetation and with a bottom of soft muck some eight inches or more deep. The *Thyone* bury themselves in this muck, leaving only the tentacles and cloacal opening exposed. They are frequently numerous and may be seen over considerable extent with one or more for every square yard.

Associated with *Thyone briareus*, in such locations are: a few of the worms, *Clymenella;* many *Lumbrinereis tenuis*, and the clam-worm, *Nereis virens*.

Of the crustacea, *Carcinides mænas*, the green crab, is most conspicuous and may frequently be seen running along the bottom, dodging in and out of the eel grass. The ever-present small hermit crabs, *P. longicarpus*, with their usual commensals, are present in some abundance together with the shrimp, *Crangon vulgaris*. The mud crab, *Panopeus*, is also found.

The molluscs are represented by *Modiolus demissus*, near the edge, where *Mya* and *Venus* also occur. There are also a few strings of *Mytilus edulis* and some rolls of *Crepidula fornicata* to be found. Mud snails, *N. obsoleta*, are also present.

TABLE XI.

SHOWING THE RESULTS OF ANALYSES OF THE WATER OVER THE *Thyone* ASSOCIATION.

All but the last two items are from NW. Gutter Flats; those are from Blind Gutter Flats.

Date 1920.	Time.	Depth in ft.	Tide.	Temp. ° C.	pH.		Oxygen.		Salin- ity.	Light.	Coll. No.
					Bot.	Sur.	Bot.	Sur.			
8/16	1:45	1.0	Ebbing	22	7.8	—	4.08	—	—	Dull	17
	2:45	0.67	Ebbing	22	7.8	7.8	—	—	—	Dull	19
8/18	12:30	4.2	Ebbing	22	—	8.0	—	—	—	Dull	27
	3:15	1.0	Ebbing	22	7.4	—	4.45	—	2.22	Rain	34
8/23	11:00	1.0	Flowing	20	7.9	7.9	4.53	—	3.01	Dull	54
	11:30	1.5	Flowing	20.5	—	—	—	—	3.01	Dull	55
	2:00	3.0	Flowing	20.5	8.2	8.2	6.20	8.37	—	Bright	59
5/24	9:15	1.0	Ebbing	21	7.8	7.8	3.96	—	3.04	Bright	62
	12:30	1.0	Flowing	24	8.0	—	—	—	—	Bright	69
	3:15	3.0	Flowing	26	—	8.5	—	—	—	Bright	76
9/3	11:30	4.5	High	17B 20S	7.4	8.2	3.23	5.51	2.88B 3.02S	Bright	110
8/25	3:45	1.0	Flowing	28	8.2	—	6.79	—	3.10	Bright	84
9/3	3:20	1.6	Ebbing	22	8.0	—	5.31	—	3.08	Bright	117

B, collection from bottom; S, collection from surface.

Except for salinity, the water over the marginal muck associa-tion shows much less range in variation than in the *S. acutus* association. Thus the recorded temperature range is 11° C. against 15°; the pH range, 0.8 *vs.* 1.7; the oxygen, 5.24 c.c. per liter against 12.97. These are the natural results of the lack of green plants in this association. On the other hand a careful (laboratory) determination of salinity, made just after a heavy shower that was one of a series running through a whole morning, showed the effects of fresh water drainage from a nearby swale by recording a salinity of 2.22 per cent. It is probable that this lessened density extended into typical *Scoloplos acutus* territory, though in a less marked form, but no tests have been made there under these conditions. Dilution after a really heavy rain would be much greater. The results from analyses of water over the *Thyone* beds are shown in Table XI.

f. The Intertidal Associations.—In this area the flats extend back to old shore lines where the rocks characteristic of shores are being gradually covered by mud accumulations; these are the typical mud flats which I have been describing. These margins, rising more or less steeply from the water, are the home of the intertidal associations. Most obviously there are two of these: that of the animals near the low tide level, and that near high tide. The former is dominated by *Mya* and is best under-stood if called the marginal *Mya* association; the latter associa-tion depends on location for its typical animals. That most studied has been the *Melampus* association.

(1) *The Mya Association.*—In the localities studied for this report, the *Mya* association is best represented along the west shore of Northwest Gutter Flats. The rocks there are an extension of those of the exposed rock association, but since they are located in the back part of the flats, mud has been deposited around them until in many places they are almost entirely covered up to the mid-tidal region. Here from well below low tide to about mid-tide the siphons of small *Mya* [1] are so numerous as to appear like stippled spots against the mud background. In the lower part, the larger siphons of *Venus* give variety to the stippling. If one try to dig up the clams, he encounters the buried rocks within a few inches of surface.

[1] Mr. G. M. Gray informs me that he has never taken *Mya* in pure muck.

Other animals to be found here are: *Natica duplicata, Nereis virens, Polycirrus eximeus*, burrowing in the mud; *Nassa obsoleta* and the hermit crab, *P. longicarpus*, with its commensals, *Hydractinia* and *Crepidula convexa*, on the bottom, retreating as the tide ebbs; and *Ostræa virginica* growing occasionally on the rocks.

TABLE XII.

SHOWING THE RESULTS OF ANALYSES OF THE WATER OVER THE *Mya* ASSOCIATION.

Date 1920.	Depth in Inches.	Tide.	Temp. ° C.	pH.	Oxygen c.c. per Liter.	Light.	Salin- ity %.	Time.	Collec- tion Num- ber.
8/16	1	Ebbing	28.0	7.5	—	Dull	—	3:15	21
8/18	1	Ebbing	22.0	7.1	—	Dull	2.22	3:00	23
8/23	1	Flowing	20.0	7.9	—	Dull	—	11:00	54
	1	Flowing	20.5	8.2 +	—	Bright	3.01	2:30	60
							3.01		
8/24	1	Ebbing	21.5	7.8	3.72	Bright	—	9:00	61
	1	Flowing	27.0	7.6	—	Bright	3.04	12:15	68
	1	Flowing	28.0	8.0	—	Bright	3.10	1:25	70
9/3	36	High	19.0	7.8	4.86	Bright	3.04	11:45	111

The temperature of the water in this association runs much higher than on the rocks; 28 degrees C. was recorded. The exposed mud at low tide must run still higher. Few oxygen tests were made here, but they showed, as was expected, that there is a good supply of oxygen in the water. In the upper reaches, at least, the water does not cover the mud long enough to lose much of its supply which came in with the high tide. Under low tide conditions the mud must contain almost no oxygen at all. The pH may be markedly lower than on the other associations, correlated with the fact that the muck is acid. The observed salinity is the same as for the marginal muck association but it probably runs lower on occasion, from the drainage after rains.

TABLE XIII.

SHOWING THE TEMPERATURE, OXYGEN TENSION, AND pH OF THE *Melampus* ASSOCIATION.

Date 1920.	Depth in Inches.	Tide.	Temp. ° C.	pH.	Oxygen c.c. per Liter.	Light.	Salin- ity.	Time.	Coll. No.
8/16	1	Ebbing	23	7.4	1.96	Dull	—	12:00	14
8/18	1	Ebbing	22	7.2	1.81	Raining	—	1:30	28

(2) *High Mud Shores* (*Melampus*) *Association.*—The last type of habitat in the series comes with the further deposition of organic mud. Under these conditions the *Mya* association would gradually be raised above low tide level and finally above mid tide level. Marsh grasses would come in and in place of the association dominated by *Mya* there would be one dominated in places by the fiddler crabs (*Uca*) and in others by the snail *Melampus lineatus*. The situations studied have had more of the latter than the former.

The *Melampus* grounds are well illustrated in the Uncatena shore of Northwest Gutter from near the bridge to the gravel spit marking the eastern side of the passage to the Bay and at various places along the other gutter creeks, especially near the rockweed association already located.

The substratum is a peaty mixture of muck and roots which is covered only at high tide and then by only a few inches to a foot or so of water. In the lower parts the mussel *Modiolus demissus* is present sometimes in abundance. At the upper side the land insects, such as the carabid beetles, are plentiful, while Thysanura are abundant over much of the region at low tide. The ground is moist at all times on account of the spongy character of the substratum and the protection furnished by marsh grasses.

Too few analyses of water of this region have been made but the low pH and accompanying low oxygen content of the water as it leaves the flat when the tide ebbs, is significant. When this water flows over the *Melampus* grounds, it would have a pH of about 8.0 and an oxygen tension of some 4.00 c.c. per L. The locality most studied would be covered with water about six hours of the twenty-four. The salinity must vary from about 3.00 per cent. at high tide to practically fresh water when low tide coincides with a heavy rain.

IV. SUMMARY OF DATA.

There are two types of data which one desires concerning the environmental complex of animals: First, what are the average conditions during the period when the divisions between habitats are most pronounced? And second, what extremes are to be

found within a given association? The available data on these points for the months of August and early September, will be presented by tables for the two series studied.

a. Rocky Eroding Shores.—The average conditions in the eroding shore series when conditions are most acute, are shown in Table XIV. This series is composed of the associations of

TABLE XIV.

ERODING SHORE SERIES.

Showing average salinity, oxygen content. temperature, and pH of different animal associations as found in collections from the bottom at low tide except for salinity where low and mid-tide data are averaged.   The last two associations are added for comparative purposes.

Ordinal ranking is based upon variation from conditions known to occur in open water.

Association.	Salinity.			O₂ c.c. per L.			Temperature.			pH.			Sum of Ranks.
	No. Rec.	Ave.	Rank.	No. Rec.	Ave.	Rank.	No. Rec.	Ave. ° C.	Rank.	No. Rec.	Ave.	Rank.	
Open....	12	3.12	1	12	5.17	1	10	21.1	2	15	8.0	1.5	5.5
Wharf pilings........	4	3.05	2	12	4.72	2	11	20.0	1	13	8.0	1.5	6.5
Exposed rocks.	6	3.02	3	8	5.91	3	11	21.4	3	9	8.16	4	13
Rockweed...... ...	4	2.99	4	6	6.33	4	6	21.5	4	6	8.13	3	15
Mya............. .	5	2.88	5	1	3.72	5	7	24.0	6	7	7.7	5	21
Melampus............	0	—	6[1]	2	1.88	6	2	22.5	5	2	7.3	6	23

the open water, wharf pilings, exposed rocks, and rockweeds growing on protected rocks. Data from the intertidal associations at the back of mud flats are added for comparisons.

The average salinity at low and mid-tides decrease in the order given. The average low tide temperatures increase in the order given from the wharf pilings but these, contrary to expectation, are lower than the open water. The average oxygen tension varies from that found in the open water by amounts which likewise arrange the series in the order given. Thus the wharf pilings gave an average of 4.72 c.c. of oxygen per liter which is 0.49 less than the average found in open water; while the exposed rock association with an observed average of 5.1 c.c. per liter is 0.74 c.c. from the open water conditions.

[1] Must range from almost fresh water during rain at low tide to about 3.00 at high tide.

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The pH averages the same in the wharf pilings and open water associations. The rockweed association comes next with the association of exposed rocks very near it. The pH values from the exposed rock association is higher than would be expected for the entire coast because only one of the three places in which it was studied was free from the influence of nearby submerged vegetation. Under entirely typical conditions the average pH in this series should likewise arrange the series in the same order as the other factors measured.

The rankings given in the table are based upon the extent of variation from conditions known to be characteristic of open water. The sum of the ordinal ranks, a poor enough method of averaging, shows a gradual increase corresponding to the ecological age of the associations.

The range of these four factors (Table XV.) tends to increase with the age of the association. This is not true of each factor taken separately, nor is the increase in total amounts in regular order, in that the rockweed association on this basis precedes the exposed rock association, when it would be expected to follow it.

In addition to the absolute range the data from these extremes show another set of relations not given in averages, that is the relative position of the extremes. In salinity of the open water the lowest record is fairly high and, while the upper extreme of salinity remains approximately constant, there is a general fall in the lower limit as one passes from open water to the older associations.

In the temperature series the characteristic change is in the matter of the greater maximum in the older associations. With pH, the young associations have practically no change, the older ones have an increased upper limit due to the action of plants, while the oldest ones show a decided decrease in the minimum on account of the acid-giving muck which is deposited in them.

A combination of the rankings based on the departure of average low tide conditions from those known to occur in open water and on amount of variation between observed extremes, places the associations in order of their ecological age, with the exception of the rocks and rockweed associations which are placed together. If in place of an index figure based on amount

of variation of extremes one substitutes the more complex index shown in Table XV., which combines the amount of variation with the location of extremes, the series is arranged by the data given here exactly in order of ecological age.

TABLE XV.

ERODING SHORE SERIES.

Showing range of variations of temperature, oxygen content, salinity, and pH at the stratum most studied in each animal association; all tide stages considered. The last two associations are added for comparative purposes.

Ordinal ranking is based on amount of variation and relative location of extremes.

Association.	Salinity in Per Cent.			Oxygen in c.c. Liter.			Temperature in Degrees C.			pH.			Ranking.		
	Limits.	Range.	Ordinal Rank.	Limits.	Range.	Ordinal Rank.	Limits.	Range.	Ordinal Rank.	Limits.	Range.	Ordinal Rank.	Sum of Ranks.	Combined Ranking.	Final Ranking.
Open.	3.02 3.14	0.12	1	3.93 5.43	1.50	1	20 22	2.0	1.5	8.0 − 8.0 +	0.01	2	**5.5**	11	**1**
Wharf pilings. . . .	2.97 3.13	0.16	2	4.06 6.00	1.94	2	19 21	2.0	1.5	8.0 8.0	0.0	1	**6.5**	13	**2**
Exposed rocks.	2.94 3.04	0.10	3	3.62 8.56	4.94	4	20 23	3.0	3	8.0 8.3	0.3	3.5	**13.5**	26.5	**3**
Rockweed .	2.97 3.02	0.5	4	4.75 8.56	3.81	3	19 23	4.0	4	8.0 8.3	0.3	3.5	**14.5**	29.5	**4**
Mya.	2.22 3.10	0.88	5	0.01[1] 4.86	4.86	4.5	19 28	9.0	5.5	7.1 8.2	1.1	5.5	**20.5**	41.5	**5**
Melampus .	slight to high tide cond.[2]		6	? high tide	high tide	4.5	high tide ?	high	5.5	7.2 high tide	5.5	high tide	**21.5**	44.5	**6**

b. The Depositing Shore Series.—The flats series intergrades more closely than the different associations of the rocks. The conditions characteristic of each association are most marked at low tide and these are summarized as averaged data in Table XVI. As might be expected the average salinity at low tide decreases regularly as one goes back on the flats. But this is the only factor considered that shows such a regular relationship. With the others the conditions over the sand bar approach those

[1] Estimated between tides.

[2] Estimated for purposes of comparison.

"Combined ranking" refers to the rankings given in Tables XIV. and XV. The final ranking is based on the preceding column.

of the open water most closely and the conditions in the inter-tidal associations at the back of the flats are normally most extreme, but in the middle regions the plant growth, mainly

TABLE XVI.

FLAT SERIES.

Showing the average salinity, oxygen content, temperature and pH of different animal associations of the flats as found in collections from the bottom, open water excepted, at low tide, except for salinity where both low and mid tides are averaged. Ordinal ranking is based upon variation from conditions prevailing in open water.

Associations.	Salinity.			Oxygen in c.c. per L.			Temperature.			pH.			Rank- ing.
	No. Collections.	Average.	Rank.	No. Coll.	Average.	Rank.	No. Coll.	Average.	Rank.	No. Coll.	Average.	Rank.	Sum of Ranks.
Open....	13	3.12	1	12	5.17	1	10	21.1	1	15	8.0	1	4
Scoloplos fragilis........	3	3.04	2	3	4.23	3	6	21.3	2	6	8.04	2	9
Phascolosoma..........	6	3.00	3	4	6.15	4	12	23.3	6	13	8.15	5	18
Scoloplos acutus........	6	2.97	4	9	3.88	5	15	23.1	5	17	7.95	3	17
Thyone..	6	2.91	5	6	4.85	2	9	22	3	8	7.9	4	14
Mya.................	5	2.88	6	1	3.72	6	7	24	7	7	7.7	6	25
Melampus.............	0	—	7[1]	2	1.88	7	2	22.5	4	2	7.3	7	25

eel grass, affects conditions so that the relationship is irregular. In the summation of ordinal rankings the effect of this confusion is shown.

The amount of range, Table XVII., also increases steadily as one passes back on the flats only in the case of the salinity measurements. The oxygen content and pH begin to increase in observed range as soon as the eel grass is encountered in the muddy sand association and reach their maximum range in the tall eel grass of the *Scoloplos acutus* grounds. The water temper-atures also increase in range but become greatest in the low water of the marginal muck association. The surface in the older associations undoubtedly becomes much warmer when exposed to air and this must be particularly marked in the *Melampus* association where the surface of the ground may be exposed to the full glare of the afternoon sun with almost no protection from the scant growth of marsh grasses.

[1] See note at bottom of Table XV.

TABLE XVII.

FLAT SERIES.

Showing range of variation of temperature, oxygen content, salinity and pH near the surface in the open water and at or near the bottom in other associations. All tide stages considered. Ordinal ranking is based upon amount of variation and relative location of extremes.

Association.	Salinity in %.			Oxygen in c.c. per L.			Temperature.			pH.			Ranking.		
	Limits.	Range.	Rank.	Limits.	Range.	Rank.	Limits.	Range.	Rank.	Limits.	Range.	Rank.	Sum of Ranks.	Combined Ranking	Final Ranking.
Open.....	3.02 3.14	0.12	1	3.93 5.43	1.50	1	20 22	2.0	1	8.0 − 8.0+	0.1	1	4	8	1
Scoloplos fragilis....	2.99 3.10	0.11	2	3.91 4.63	0.72	2	20 24.5	4.5	2	7.6 8.25	0.65	2	8	17	2
Phascolosoma......	2.97 3.10	0.13	3	4.21 10.39	6.18	4	21 27	6.0	3	7.7 8.5	0.81	3	13	31	3
Scoloplos acutus.....	2.88 3.02	0.14	4	trace 9.31	9.50	5	18 26.5	8.5	4	7.3 8.45	1.15	4	17	34	5
Thyone....	2.22 3.10	0.88	5.5	3.23 5.31	2.08	3	17 28	11.0	5	7.4 8.2	0.8	5	18.5	32.5	4
Mya......	2.22 3.10	0.88	5.5	0.0[1] 4.86	4.86	6.5	19 28	9.0[2]	6	7.1 8.2	1.1	6	24	49	6
Melampus.	slight to high tide cond.		7	? high tide		6.5	high tide ?		7	7.2 high tide		7	27.5	52.5	7

The relative position of the extremes in salinity is much the same as in the eroding shore series. At high tide the salinity is practically constant throughout, but at low tide the lower limit decreases regularly with distance from the open water. The relations of oxygen content, pH, and temperature at the bottom are exactly similar to those of the preceding series except that they are more striking.

The ranking in this series, as in Table XV, is based upon the amount of range combined with the relative location of the extremes. On this basis salinity, temperature, and pH arrange the series in order of the ecological age of the different associations. On the basis of oxygen content, the marginal muck association is much younger than is expected. The combination

[1] See note at bottom of Table XV.
[2] Water temperatures only considered.

of these rankings arranges these associations in order of their age but when combined with the rankings from average conditions shown in the preceding table, the marginal muck (*Thyone*) association is placed slightly earlier in the series than it belongs. Further data would probably adjust this arrangement.

At the surface of this series, the range of oxygen, pH and temperature is greatest in the *Scoloplos acutus* region. The mat of eel grass just at the surface at low tide allows a surface layer of very warm water to be found in the bright sunlight where it is supersaturated with oxygen and has a correspondingly high pH. The *L. litorea*, *S. luciæ*, *Bugula*, *C. convexa*, *B. alternatum*, *Molgula*, and *Botryllus*, which occupy this region must be very resistant to these extreme conditions. Under the most pronounced conditions few animals capable of moving are found at the surface.

The differences on the flats level off at high tide. The tide appears to come to the back of the flats over the surface of the more stagnant water which has remained behind during low tide, bringing lower temperature, higher salinity, lower oxygen content and lower pH. Thus two collections from the back of the flats in 4.5 feet of water on a flowing tide showed a specific gravity of 1.018 at the bottom and 1.022 at the surface. Obviously such conditions do not prevail long and by diffusion the gradients disappear. In the long eel grass (*S. acutus* association), the vertical gradient at low tide in one foot of water was found to be from 3.37 c.c. oxygen per liter to 10.39 at the surface. Three hours later in the same place in three feet of water of the new tide the gradient was from 7.03 at the bottom to 8.3 at the surface. Similarly a pH gradient of from 7.7 at the bottom and 8.7 at the surface became one of 8.0 to 8.1.

The condition of the water over the bottom of the flats at high tide tends to become uniform throughout all the associations. Thus at NW. Gutter Flats, collections made under typical high tide conditions showed a range of 0.1 in pH over the flats while at low tide the range at similar stations was over 1.2.

V. Discussion.

Sumner (1908) in discussing the study of the distribution of bottom living animals in the Woods Hole region considered

character of bottom, depth, temperature, salinity, purity of water and currents as the important factors in determining distribution. The first of these factors, character of bottom, is characteristically different in the associations of eroding and depositing shores. The former is characterized by the presence of firm places for attachment and difficult burrowing conditions; the latter, by the converse of these conditions. The substratum also serves largely in distinguishing between the different associations of each series.

The influence of depth as a factor in animal distribution in the region covered by these studies is not due to depth as such but to depth as assuring a constant supply of water. This is shown in the rockweed association where animals normally found below low tide level on the wharf pilings may live well above it when protected from drying and from high temperatures by the mat of rockweeds.

Temperature serves as a limiting factor for these associations, during the season of the year studied, in the tide pools and more particularly on the flats. There in the *Scoloplos acutus* association above the dense eel grass mats at low tide the high temperature (32° C.) that may be reached must serve to kill off the more sensitive sessile animals as it drives the motile ones below the surface layer to the cooler water in the shade of the eel grass. The temperature to which animals may be exposed at low tide increases as one leaves open water conditions in both series. The effect of this high summer temperature of the flats as a factor limiting the geographic distribution in this region will be discussed in Study IV.[1] of this series.

In the associations studied, the salinity regularly increased as one approached open water conditions. The low salinity on the back part of the flats particularly in the *Melampus*, *Mya* and *Thyone* associations must serve as a limiting factor. These are subjected to such extreme ranges of salinity following heavy rains, particularly if these rains come at low tide and where there is some considerable surface drainage, that sensitive animals or animals in a sensitive stage in their life history must needs be killed or driven off.

In the locations studied, there was no contamination from

[1] In press in *Ecology*.

sewage wastes, so this possible factor in distribution may be dismissed.

The effects of tidal currents are well illustrated in the gutters and creeks of Hadley Harbor. These protected channels, supplied with constantly changing water which differs from open water only by the effect of coming over large tracts of submerged vegetation, support a wholly different animal life from that present where such currents do not enter. These locations are in the exposed rock or the rock-rockweed stage of development, in place of the *Mya* or *Melampus* stage which they would occupy if the currents were absent. In addition to these scouring effects of tidal currents, they have the well known function of oxygen and food carriers. They also eliminate the depth gradients in oxygen and pH found commonly in the stagnant water of the older associations.

The relation of oxygen and pH of the sea water to animal distribution has received no attention in the Woods Hole region. Both depend (1) on the supply of offshore water, (2) the amount of photosynthesis being carried on nearby, and (3) the character of the bottom. In the open water these factors depend upon currents and the proximity to vegetation. In the presence of vegetation oxygen is given off and the pH is increased. Muck absorbs oxygen and lowers the pH while sand has no effect on either unless it has been laid bare, when it, as well as rocks under similar conditions, decreases the pH without affecting the oxygen supply. Wave action has the converse effect.

From examining data from such regions as the rockweed or eel grass one might be tempted to generalize and say that as the oxygen increases the pH likewise increases and *vice versa*. Such a conclusion at best holds only in regions of abundant vegetation or of muck, and there the pH changes lag behind changes in oxygen concentration.

If the invertebrates are as sensitive to pH variations as Powers found herring to be, this correlation of high hydrogen ion concentration with the low oxygen regions of the muck must serve to keep free moving animals out of such conditions. Such action, combined with its greater regularity of distribution and slower fluctuations, makes the pH of the water more important

in such studies as the present than is the distribution of oxygen.

Both factors, however, may range widely within a given association. Take for example the sensitive *Amarœcium* association which flourishes on wharf pilings or on exposed rocks. At the mouth of Northwest Gutter, where the pH stays about 8.0 and the oxygen ranges from 3.72 to 4.95 c.c. per L., the rocks support a typical rock-*Amarœcium* association. Yet the same association, fully as rich in species, occurs at Gansett on similar rocks, but with much plant life all about and with the pH varying from 8.0 to 8.3 and the oxygen from 5.15 (and probably lower) to 8.56 c.c. per L. It will be noted that the range of conditions either at Northwest Gutter or at Gansett is lower than when the two are combined, so that, while the association can exist in these limits, it is not subjected to these extremes in one locality. The total range in both temperature and salinity is likewise greater when the two locations are considered together than when either is taken singly.

It is probable that a collection of data concerning the conditions under which the different sensitive animals live in all their different localities would, when thrown together, indicate that they could stand widely differing concentrations of all the water factors considered; when, as a matter of fact, they are exposed to relatively slight changes in the location in which they do live.

With less sensitive animals the association limits as set out in this paper mean nothing. *Pagurus longicarpus* apparently roams at will in all of those of the flats and occurs among those of the rocks, carrying with him, willy-nilly, his commensals. The mud snail, *Nassa obsoleta*, is at present found among all the associations, from the clean sand to the inter-tidal associations, and, according to Dimon, originally dominated the sand also before being driven off by *Littorina litorea*. In part it is able to do this because it is a resistant animal and in part on account of the fact that it probably becomes accustomed to conditions in a given locality and tends to keep within them. In this regard, one can but express the wish for more studies like that of Dimon. With a series of such studies at hand one could draw definite conclusions where now, so far as individuals are concerned, he is limited in large part to theorizing.

There is so much work necessary in making an ecological

survey that there is always a lively interest in single factor indices of associations, and at the beginning of the present studies I rather expected to find such an index in the pH relations. While a combination of the average pH, the extent of range and the relative position of the extremes does allow one to place these associations in their natural order with considerable exactness, and while such data is very suggestive it does not classify these associations with the precision necessary for a successful single factor index. This is emphasized in the rock series of associations, Tables XIV. and XV.

The use of the oxygen content of the water is out of the question as such an index of an association. Temperature is an aid but needs confirmation. Salinity, whether average or range, considered with position of extremes, does arrange the different associations in their logical order, and so qualifies for the recommendation that was predicted for pH, as the best single index when water conditions alone are considered. The readily determined factors of salinity, temperature and pH taken together give a much stronger index than any one of them alone.

If, however, on account of urgent haste, I should be forced to make use of a single criterion to divide the communities of the Woods Hole littoral, I should depend more on observation of the character of the sea bottom than on any other one factor.[1] This, the most obvious, the longest used, is still the least treacherous single factor index of littoral distribution in this region. It should be used with discretion since a rock well back on the flats supports a different set of animals from one on an exposed point, but the corrections are more obvious and more easily applied.

All the data collected in the present investigation agree in supporting the common sense conclusion that animal associations in a region such as this under consideration are not normally limited by any one factor, but by the interaction of several, and when feasible all these should be analyzed and recorded.

[1] Shelford ('14) in writing of the suitability of water for fishes concluded that the amount of clean bottom, the amount of carbon dioxide and the amount of hydrogen sulfide, taken together serve as an index of availability of bays and enclosures of the seas for fish life. Longley ('22) in studying the local distribution of Tortugas fishes concludes that the local distribution of many species is determined by the character of the bottom. This holds particularly for what he calls the "sand-patch" association.

The ecological age of the different associations has been repeatedly mentioned. This idea clearly and repeatedly stated by such workers as Cowles, Shelford and Adams, is apparently not yet fully understood. Briefly, it means that the *Melampus* association as it exists at present is an old association which has passed successively through the other stages standing before it in the series. Thus at one time the spot on which a *Melampus* association is now located was bare sand, which, as it became finely ground and somewhat packed, began to support a *Scoloplos fragilis* colony much as is found on Blind Gutter Bar at the present time. With accumulation of the organic products of these animals in the absence of a scouring current, other animals came in until the *Scoloplos fragilis* association began to resemble a *Phascolosoma* association. As more muck was deposited the older associations were passed one by one until the present old *Melampus* association resulted. With further deposition the land will be raised above tidal level and the *Melampus* association will gradually give way to strictly land animals. In this region the muck that accumulates is almost entirely of organic origin (cf. Survey, p. 32) so that the animals themselves have played a considerable part in causing ecological succession to take place.

Transitional stages between the different associations are abundant. A particularly noticeable one was studied that belongs between the *Scoloplos acutus* and *Thyone* associations. This is located near the gravel spit at the Uncatena side of the Northwest Gutter passage. There the *Scoloplos acutus* occur in the muck, but *Thyone* are absent as yet, although they are found nearby in greater numbers each year. Of the eight comparisons that have been made in the summary tables, four would place this location with the *Thyone* association, three place it as younger than *Phascolosoma* association, and the other with *Scoloplos acutus* where it belongs according to its animal inhabitants, and according to the average of these physical factors.

The wharf pilings present a specialized *Amarœcium* type of association that is obviously younger than the rock-*Amarœcium* community, although closely related to it both in animals present and in physical conditions. It represents more nearly the type of habitat that might be found where large rocks extend up out

of deep inshore water. Being a man-made habitat it is not, strictly speaking, a part of the rock series and is included in that series because it does give approximately the same condition that would be found on the rocky pillars just mentioned.

The succession of forms in this association can readily be studied. Glass slides placed under wharfs furnish a convenient method of finding the first pioneers to be expected. Observation of the growth of communities on new non-creosoted pilings and comparison with those on middle aged and old pilings would give the whole story for the wharf pilings, since they will of course never become a *Mya* association, as a rock-*Amarœcium* association may, or in the same way that its rock pillar prototype might.

In addition to such a study as this and to the studies of the ecology of individual species, in order to describe completely the littoral ecology of this region, studies should be made in the late autumn, early spring and in late spring or early summer. This last is particularly needed to round out our knowledge of the physical and faunistic conditions in these associations during the spring reproductive period.

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BIOLOGICAL BULLETIN

CERTAIN EFFECTS OF THE SALTS OF THE HEAVY METALS ON THE FERTILIZATION REAC-TION IN *ARBACIA PUNCTULATA*.

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I. INTRODUCTION.

At the suggestion of Prof. F. R. Lillie in the spring of 1921, I began, the following summer, a study of the effects of the heavy metals on the fertilization reaction in *Arbacia punctulata*. I desire here to express my appreciation for his many helpful suggestions and his aid in the interpretation of the results.

Lillie ('21) found that copper salts completely prevent the elevation of fertilization membranes in *Arbacia* even at such a great dilution as 1 : 500,000, though the concentration of copper chloride required for the suppression of cleavage is 1 : 62,500. It is the purpose of the present set of tests to establish the effects of a series of metallic salt solutions on the fertilization reaction and by this means to throw more light on the initial events of this reaction. The work was done during the summers of 1921 and 1922 at the Marine Biological Laboratory at Woods Hole, Massachusetts.

The experiments brought out very clearly that there is a sharp distinction between the initial and the subsequent events

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of the fertilization reaction. As soon as the initial part of the reaction is completed, *i.e.*, as soon as the membrane has raised, the metallic concentration which has formerly been inhibitory in its action ceases to be wholly effective and some of the eggs may develop as far as the motile free-swimming forms in the solution. In the following pages, such a concentration, which inhibits the elevation of membranes, when eggs are inseminated in it, will be termed "membrane-inhibitory." Such inhibition of membrane elevation is complete. If, however, the concentration of the salt is greatly increased, a concentration may be reached at which cleavage in turn is completely inhibited, but here again, the time factor is important. For example, if a given concentration is toxic to cleavage for a set of eggs transferred to it five minutes after insemination in normal sea water, it is said to be "cleavage-toxic." It may allow 40 per cent. to 50 per cent. cleavage if eggs are transferred to it 20 minutes after such an insemination, or even 95 per cent. if they are transferred after a 30-minute interval. In such a case, second cleavage will be completely inhibited. When cleavage is used as a criterion of toxicity, the period of exposure to the solution will vary according to the length of time elapsing between insemination and transfer. If, when eggs are transferred to the solution five minutes after insemination, first cleavage is inhibited, then it follows that eggs transferred at a later time will not perform second cleavage, though they may cleave once.

McGuigan ('04), v. Euler and Swanberg ('21), Olsson ('21), and others have investigated the effects of the salts of certain of the heavy metals on enzyme action. Mathews ('04), working with *Fundulus*, compares the physiological action of the heavy metals with their solution tensions. It was with the same idea that McGuigan worked on the ferments. He found that, generally speaking, a low solution tension was indicative of great toxicity. There were, however, certain exceptions to the series. v. Euler and Swanberg attribute the poisonous effect of the salts to their property of binding certain groups of the enzyme molecule and thus making it ineffective.

In the ferment experiments just cited as well as in the experiments made by Mathews on *Fundulus*, the experiments allowed

the use of distilled water in the solutions, and in consequence, the dissociation of the salts could be accurately measured and precipitation did not trouble the investigators. In the work with *Arbacia*, on the other hand, it was necessary to use sea-water for all of the final solutions though the stock solutions were made in distilled water. The composition of the sea-water is of such a nature that in many cases there is a precipitation and in no case could the ionization of the salt be accurately determined. The sea-water, as it comes from the tap in the laboratory, has a p.H. of 8.0 and I may say here, that in no case did the concentration of the metallic salt necessary to inhibit membrane formation have a p.H. below 7.0. In some of the solutions there was a visible precipitate, as was most evident in the cases of Zinc, Lanthanum, and Lead. In such solutions, the balance of the salts must be disturbed. Thus, in the case of the alkaline earth metals, and silver, tin, manganese, chromium, and iron, the precipitate was so heavy that after a few preliminary experiments, their use was abandoned. The precipitation of zinc, lanthanum, and lead was not great enough to hide the effect of the salts on the elevation of the membranes but when the concentration was increased for the investigation of cleavage-toxicity, the precipitation was so heavy that it was deemed unwise to try to determine that point for them.

When the series was completed, it was found that the work included tests made with the following metals: Gold, copper, zinc, lanthanum, aluminum, platinum, lead, nickel, cadmium, cobalt, and mercury. The results of the experiments show a similar behav or of the metals, save mercury, and those described by Lillie ('21) for the chloride of copper. The inhibiting concentration varies for the different metals.

The behavior of mercuric salts is peculiar and unexplainable at present. Its solutions seem to favor membrane elevation up to a concentration at which the sperm are immediately paralyzed, while at a much greater dilution, cleavage is completely inhibited. This will be treated in more detail in the following pages.

Rhubidium and cesium were found to be indifferent up to the isotonic concentration and will not be treated further.

II. The Normal Fertilization Reaction in

Arbacia Punctulata.

In order to understand the later discussion, it will be wise here to summarize the phenomena of the normal fertilization reaction in *Arbacia*. These were described in great detail by Lillie ('14), though some of the events had been incompletely described by von Dungern ('02). Fol ('79) described the penetration of the spermatozoön and the elevation of the fertilization membrane in a classical monograph.

Before the spermatozoön comes into contact with the egg itself it is activated,and directed toward the egg as described by Lillie ('13). The egg, from the time of rupture of the germinal vesicle up to the time of fertilization, or, when fertilization is barred, up to the time of cytolysis, produces an iso-agglutinin (Lillie '13) or fertilizin (Lillie '14) which produces a reversible agglutination of the spermatozoa to the egg. Between the time of the agglutination of the spermatozoa to the egg and the beginning of the fertilization reaction, there is a latent period which is very short but variable in *Arbacia*. This is followed by a very rapid initial reaction which produces a sterilization of the egg against other spermatozoa, described by Just ('19) as "a wave of negativity." The penetration of the spermotozoön occurs immediately, followed by the elevation of the membrane. In a given lot of fresh eggs, the time elapsing before membrane elevation is very variable. At 19.5° C. some eggs appear with membranes fully formed in from 15 to 30 seconds while others require much longer. Elevation of the membrane is too rapid for observation in *Arbacia* and it seems probable that the variable is due to the latent period. With stale gametes the process may extend over five minutes or more and is often accompanied by polyspermy.

III. Methods.

The experiments described in the following pages are very simple. Eggs were removed from good ripe females by making a circumferential cut around the animal and removing the gonads intact to about 150 c.c. of sea water in a finger bowl. The ovaries were then cut a number of times and the ova allowed

to stream forth.   In about five minutes, the suspension was strained through cheese cloth to remove the pieces of the ovaries. The eggs were then allowed to settle.   When they had settled, the supernatant liquid was poured off and the dish again filled with fresh sea water.   This process was repeated three times so that all traces of the coelomic fluid and the tissue juices were practically eliminated.   Eggs were not used unless the normally fertilized control showed above 95 per cent. membrane formation and a good viability through the first cleavage.

Males were cut in the same way and placed in a Syracuse glass with the genital pores down.   The sperm flows from the pores in a creamy mass, which, when mixed with sea water in the proportion of one drop of dry sperm to 25 c.c. of sea water gives a grayish, opalescent suspension which is described in this paper as a 1 : 25 sperm suspension.   One drop of this mixture when added to one drop of eggs in 7.5 c.c. of sea water gives 100 per cent. membrane formation with no polyspermy and such an insemination is spoken of as a 1 : 25 : 7.5. insemination.

Concentrated stock solutions were made up as percentage of metallic salt in distilled water by means of the volumetric flask. The solutions used in the experiments were made up by the addition of the stock solution to sea water.   Care was taken with those metals which required high concentrations, that the proportion of sea water and stock solution in the mixture used should not alter the osmotic pressure to any appreciable extent. Solutions were not used for more than five days save in the case of $AuCl_3$ and $PtCl_4$ where the rarity of the metal demanded the use of the one solution.

When an insemination is spoken of as immediate, *i.e.*, made in the solution itself, 7.5 c.c. of the solution was put into a watch crystal and one drop of eggs and one drop of 1 : 25 sperm suspension added at opposite sides of the dish.   The dish was immediately rotated to insure thorough mixing of the gametes. In the viability tests, the eggs were normally inseminated in sea water and one drop of these was then added to 7.5 c.c. of the solution.   The dish was then rotated as stated for the immediate inseminations.

A number of comparative tests were made in which, owing to

the size of the experiment set up, it was necessary to use the eggs of a number of females. Unless so stated, each experiment was made with the eggs and the sperm of but one pair of individuals.

IV. Experiments.

I have chosen to deal with the experimental section of this paper in three parts. In the first I will take up in some detail the behavior of each solution which I used. I have taken the metals up in the order of decreasing toxicity. It will be noted that Mercuric chloride is apparently out of place, but as it gives no membrane inhibiting point in the same sense as the other metallic salts, it has been put at the end.

The series of tests described in part b was made for the purpose of comparison. The tests show the great similarity in the action of the various salt solutions on the eggs and the sperm, not only when they are unmixed, but also on insemination. The latter includes both immediate inseminations and viability tests.

Under c I have summarized the experimental data. There is also a table there that shows both the membrane-inhibiting concentration and the cleavage-toxic concentration for each of the metals.

a. Data on the Various Metallic Salts.

1. *Gold Chloride.*—On July 20, 1922, a 1 per cent. stock solution of $AuCl_3$ was made in distilled water and used for establishing concentrations in sea-water as shown in Table I*a*. These were prepared separately for, and at the time of each experiment. A series of tests was then set up to establish the effects of various concentrations on eggs which were inseminated immediately in the solutions. The results are shown in the accompanying Table I*a*. It can be seen on examination, that one part of $AuCl_3$ to 1,500,000 parts of sea-water will completely inhibit membrane elevation. At 1 : 600,000 and 1 : 300,000, narrow hyaline zones appear around the eggs after an exposure of about five minutes to the solution, but upon examining the control, it was found to be merely the first indicium of cytolysis in the egg.

A series of tests was then made with the purpose of determining what concentration is necessary to inhibit cleavage when the eggs are transferred to the solutions five minutes after insemina-

TABLE I*a*.

Concentration of AuCl₃ in Sea-water.	Per Cent. Membranes.		Per Cent. 1st Cleavage.	
	♀ A.	♀ B.	♀ A.	♀ B.
1 : 12,000,000	100	100	99	100
1 : 6,000,000	68	20	69	18
1 : 3,000,000	22	2	20	2
1 : 1,500,000	0	0	0	0
1 : 1,200,000	0	0	0	0
1 : 600,000	0	0	0	0
1 : 300,000	0	0	0	0
Uninseminated control in sea-water...	0	0	0	0
Inseminated control in sea-water.....	100	100	100	100

Effect of AuCl₃ on eggs inseminated therein.   July 20, 1922.   Temp. 22.5° C. Eggs and sperm fresh.   Insemination immediate 1 : 25 : 7.5.

tion in normal sea-water.   The results for one of these tests is given in Table I*b*.   Table I*b* shows the concentration of AuCl₃ toxic to cleavage to be 1 : 375,000.   Comparison with Table I*a* brings out the difference between membrane-inhibiting and cleavage-toxic concentrations.

TABLE I*b*.

Concentration of AuCl₃ in Sea-water.	Per Cent. 1st Cleavage.	Per Cent. 2d Cleavage.
1 : 1,500,000	90	3
1 : 750,000	12	0
1 : 375,000	0	0
1 : 185,000	0	0
Inseminated control in sea-water....	98	97

Test of cleavage-toxicity of AuCl₃.   July 20, '22.   Eggs from ♀ A.   Opened 1 : 34 P.M.   Membranes 100 per cent.   Eggs transferred to solution five minutes after insemination in normal sea-water.   1 : 25 : 7.5.   Sperm fresh.   Temp. 24° C.

In Table I*c* can be seen viability tests run with the same lot of eggs at both the cleavage-toxic and the membrane-inhibitory concentrations.   This test shows the difference between these two kinds of action.   A concentration that is completely inhibitory to the initial events has no absolute effect on the subsequent events, while a more concentrated solution will prevent the occurrence of the subsequent events provided the time factor is sufficient.   The effect of the exposure time is also shown by this table.   The effect on cleavage is cumulative.   The higher the concentration of the metallic salt, the shorter need the time of exposure be, to produce complete inhibition.

TABLE Ic.

Time of Transfer after Insemination in Normal Sea-water.	Per Cent. Membranes.		Per Cent. 2 Cell.		Per Cent. 4 Cell.	
	A.	*B.*	*A.*	*B.*	*A.*	*B.*
Immediate.	0	0	0	0	0	0
30 sec.	92	93	29	0	12	0
1 min.	95	95	46	0	14	0
2 "	98	98	80	2	65	0
4 "	100	100	84	4	68	0
8 "	100	100	84	16	80	0
15 "	100	100	92	24	86	0
30 "	100	100	96	78	95	0
Inseminated control in sea-water.	100		98		99	

Viability tests. Aug. 12, '22. 5 : 27 P.M. Temp. 21° C. Eggs ♀ *B.* 5 : 10 P.M. Sperm fresh. Insemination 1 : 25 : 7.5. Solutions:

A, 1 : 1,500,000 AuCl₃.

B, 1 : 375,000 AuCl₃.

Li lie ('21) found that there are three ways in which egg when placed in the membrane-inhibiting concentration of CuCl₂ can be made to fertilize and produce membranes. Two of these are by means of protective agents. In the first case he used egg sea-water of high agglutinating power. In the second, he used a solution of gelatine or of gum arabic. The third method is by the use of an excess of sperm. A test was made of the effect of increased sperm concentrations on gold inhib:tion with the results shown in Table Id. A concentration of AuCl₃ of

TABLE Id.

Drops 1 : 25 Sperm.	Per Cent. Membranes.	Per Cent. 1st Cleavage.
1	0	0
2	0	0
4	0	0
6	4	3
8	20	20
12	36	34
20	100	92 much polyspermy.

Effect of increased sperm concentration. Aug. 7, 1922. 10 : 38 A.M. Temp. 25° C. Eggs 9 : 50 A.M. Sperm fresh. AuCl₃ 1 : 750,000.

1 : 750,000 was used in this experiment. Such a concentration has a p.H. of 8.0 and has no visible effect on the spermatozoa.

2. *Copper Chloride.*—Lillie ('21) investigated the effect of

copper salts of the fertilization reaction of *Arbacia* and found that there was a membrane-inhibiting point at 1 : 500,000   He also determined the cleavage-toxic concentration as 1 : 62,500. A repetition of this work confirmed the determinations that he made save that for one of the females employed it was found necessary to use a concentration of 1 : 37,500 $CuCl_2$ to completely inhibit cleavage. As has been stated above, the cleavage-toxic concentration varies somewhat, both for the eggs of different females and for the different physiological cond.tions existing in the eggs of the same female.

3. *Zinc Chloride.*—Zinc, it will be recalled, is one of the metals which formed such a precipitate that a cleavage-toxic concentration could not be determined. In the weaker solution necessary to completely inhibit the formation of membranes, however, the salt could be used with little difficulty. The usual series of experiments for the determination of this point was set up. The result of such an experiment may be found in Table IIa.

TABLE IIa.

Concentration of ZnCl₂ in Sea-water.	Per Cent. Membranes.	Per Cent. 2 Cell.	Per Cent. 4 Cell.
1 : 250,000	96	76	Present.
1 : 200,000	6	5	"
1 : 175,000	0	0	0
1 : 150,000	0	0	0
1 : 125,000	0	0	0
Inseminated control in sea-water	96	96	96
Uninseminated control in sea-water	0	0	0

Test of membrane-inhibition of $ZnCl_2$. July 5, '22. ♀ C. Eggs and sperm fresh. Insemination 1 : 25 : 7.5. Exp. 2 : 30 P.M. Insemination immediate. All membranes narrow.

Table IIb is a record of the viability of eggs which were inseminated in normal sea-water and transferred to the $ZnCl_2$ solution at intervals as indicated. It will be noted that when good membranes are raised, the toxicity of the membrane-inhibiting concentration on cleavage is not very marked. When narrow membranes have been raised the viability is much poorer.

4. *Lanthanum Chloride.*—Gray ('15) found that cerous chloride in a very weak concentration, would aggregate the sperm of *Arbacia*. He also found that by the addition of a small amount

TABLE II*b*.

Time of Transfer after Insemination in Normal Sea-water.	Per Cent. Membranes.	Per Cent. 2 Cell.	Remarks.
Immediate........................	0	0	
30 sec...........................	95	63	33 per cent. narrow membranes.
1 min...........................	100	82	Memb. good.
2 "	100	98	" "
2 "	100	98	" "
4 "	100	100	" "
8 "	100	100	" "
15 "	100	100	" "
30 "	100	100	" "
Inseminated control in sea-water......	100	100	

Viability test. 1 : 150,000 ZnCl₂. July 5, 1922. Eggs and sperm fresh. Insemination 1 : 25 : 7.5. ♀ A. Exp. 2 : 45 P.M. Temp. 20° C.

of NaOH, the sperm could be reactivated. Because of this, I made a few additional experiments on the sperm of *Arbacia* with LaCl₃ solutions. In the case of the immediate insemination in the lanthanum chloride solution, I found that, at a concentration of $N/4,090$ or 1 : 50,000, membranes are not raised. I then made up a thick sperm suspension by adding a number of drops of dry sperm to 7.5 c.c. of the LaCl₃ solution and observed that the sperm clumped and that their activity was much inhibited. The sperm in this solution were, however, reactivated by the addition of a drop of $N/500$ NaOH. I then made a similar suspension and added one drop of concentrated egg sea-water. The sperm immediately became very active and gave a very definite agglutination reaction.

After the membrane-inhibitory concentration had been established as 1 : 50,000, this solution was made up replacing the normal sea-water with concentrated egg sea-water, *i.e.*, egg sea-water of high agglutinating power. When eggs were inseminated in such a solution, the percentage of membrane formation rose from 0 per cent. to 64 per cent. to 72 per cent. It may thus be seen that egg sea-water has a great protective action in lanthanum-inhibition.

The viability test of the normally inseminated eggs in 1 : 50,000 LaCl₃ solution showed that this concentration is very slightly toxic to cleavage. As will be recalled, a cleavage-toxic concentration could not be determined because of the great amount of precipitation.

5. *Aluminium Chloride.*—The tests with AlCl₃ were much·like those for the metals already described. The results were also much the same. It was found that a concentration of $1 : 40,000$ (p.H. 7.2) would completely inhibit membrane elevation and that a concentration of $1 : 10,000$ (p.H. of less than 6.6) was toxic to cleavage when the eggs were transferred to it after insemination in normal sea-water. As in the case of LaCl₃, the sperm were clumped when added to the solution, but when NaOH or egg sea-water was added, this clumping was corrected and the sperm became active. When the egg sea-water was added, the sperm agglutinated. Table IV. shows the result of

TABLE IV.

Time of Transfer after Insemination in Normal Sea-Water.	Per Cent. Membranes.	Per Cent. 2 Cell.
Immediate..........................	0	0
30 sec.............................	80	20
1 min..............................	97	80
2 "	97	92
4 "	97	94
8 "	97	97
15 "	97	96
30 "	97	97
Inseminated control in sea-water........	97	98
Uninseminated controls.		
a. Sea-water...................	0	0
b. AlCl₃......................	Eggs clumped.	

Viability test in $1 : 40,000$ AlCl₃. June 19, 1922. ♀ *A.* Eggs and sperm fresh. Insemination $1 : 25 : 7.5$. Exp. $1 : 00$ P.M. Temp. 21.5 C.

a viability test on eggs inseminated in normal sea-water and later transferred to the membrane-inhibiting concentration of the salt. It will be noticed by an examination of this table, that the membrane-inhibiting concentration is more toxic to the first cleavage than is the case with the other metallic salts.

A test of the increase of membrane elevation due to an increase of sperm concentration showed that the percentage was raised from 0 per cent. to 32 per cent. on increasing the amount of sperm from 1 drop to 15 drops of the $1 : 25$ suspension in immediate inseminations.

6. *Platinic Chloride.*—In the case of no other metallic salt studied is there as sharp a line of demarkation between concentrations which permit of membrane elevation and those which are absolutely inhibitory, as with PtCl₄. A stock solution was

made from the crystals which was .9 per cent. in strength and all the concentrations were made up by adding this to sea-water. The test for the membrane-inhibitory concentration shows that at 1 : 16,666, there is membrane elevation to equal the control, while at 1 : 11,111, there is a complete inhibition of membrane elevation. The p.H. of the latter concentration is 7.4. In this case each egg may be surrounded by from twelve to sixteen very active sperm. They strike the membrane and remain agglutinated to it though they are unable to incite any cortical change whatever. The agglutination of the sperm, by a drop of egg sea-water, in such a $PtCl_4$ solution when the suspension is made up directly in the solution, is good. The inhibition comes after the agglutination and before the cortical response of the egg can show itself, *i.e.*, it occurs in the latent period.

Not only is there a sharp distinction between concentrations which allow complete membrane elevation and those which are absolutely inhibitory, in the case of this metal, but the membrane-inhibiting concentration and the cleavage-toxic concentration are both more rapidly toxic to the eggs than is the case with the other metals studied. Table V*a* shows the results of a viability

TABLE V*a*.

Time of Transfer after Insemination in Normal Sea-water.	Per Cent. Membranes.	Per Cent. 1st Cleavage
Immediate..........................	0	0
30 sec..............................	88	44
1 " ....,....	92	50
2 "	96	62
4 "	98	84
8 "	98	84
15 "	98	98
30 "	98	97
Inseminated control in sea-water......	98	98
Uninseminated control in sea-water.....	0	0

Viability test in 1 : 11,111 $PtCl_4$. June 30, 1922. Eggs 9 : 00 A.M. ♀ *A*. Sperm fresh. Insemination 1 : 25 : 7.5. Experiment 10 : 00 A.M. Temp. 21° C.

test at the membrane-inhibiting concentration of this salt; Table V*b* shows the effect of such a solution on eggs which have been forced to produce membranes by the use of an excess of sperm; Table V*c* shows the result of a viability test at the cleavage-toxic concentration. All of these tests show the great poisonous effect of the solution on the egg. The test with the

TABLE V*b*.

Drops of 1 · 25 Sperm Suspension.	Per Cent. Membranes.	Per Cent. 1st Cleavage.
1.....................................	0	0
2.....................................	0	0
4.....................................	2 narrow.	0
8.....................................	70 "	0.5 irregular.
12.....................................	80 "	0
16.....................................	100 "	1
Inseminated control in sea-water.......	100 good.	100
Uninseminated control in sea-water.....	0	0

Effect of increased sperm concentration in immediate insemination in 1 : 11,111 PtCl₄. June 30, 1922. Eggs as in Table V*a*. Exp. 10 : 00 A.M. Temp. 21° C.

increased sperm concentration shows the resistance of this solution to enforced insemination as well. Only after the eggs have been left in normal sea-water for four minutes after insemination therein, does the viability in Table V*a* begin to approach the normal control. At this same concentration, 1 : 11,111, while 100 per cent. membrane elevation may be produced by increase of the sperm concentration from one to sixteen drops of the 1 : 25 suspension, only 1 per cent. of the eggs cleave and these are irregular.

TABLE V*c*.

Time of Transfer after Insemination in Normal Sea-water.	Per Cent. 2 Cell.	Per Cent. 4 Cell.
10 min...........................	0	0
20 "	0	0
30 "	0	0
45 "	2	0
Inseminated control in sea-water.......	96	96

Viability test in 1 : 1,945 PtCl₄. July 1, 1922. Eggs 2 : 00 P.M. Sperm fresh. Insemination 1 : 25 : 7.5. Membranes 100 per cent. Experiment 3 : 30 P.M. Temp. 22° C.

A test of the cleavage-toxic point gave that determination as 1 : 1945. As soon as the eggs were transferred to such a solution after insemination in normal sea-water, they become crenate. Such a solution allowed no cleavage up to 45 minutes when the eggs were transferred to it at intervals after insemination in normal sea-water. The results of this experiment are given in Table V*c*. Cleavage took place in 48 minutes in the controls.

7. *Lead Chloride.*—PbCl₂, when added to sea-water precipitates very freely. This precipitate is not so heavy, however, that the membrane-inhibiting concentration cannot be determined as 1 : 4,000 or $N/554$ (p.H. 7.0.). Some precipitate was present

even at this concentration so that the amount of the salt really in solution could not be figured. Sperm will agglutinate readily in such a solution, though the eggs become clumped.

8. *Nickel Chloride.*—A series of tests, made with $NiCl_2$, follow so closely the general behavior of the metallic salts already described that but little discussion is necessary here. It was found the membrane inhibition first becomes evident at about 1 : 30,000 when the percentage of membranes raised fell from 100 per cent. to 80 per cent. in immediate inseminations, while at 1 : 5,000, $N/324$, membrane elevation is completely supressed. Such a solution, when used in a viability test, shows a decided effect on the production of cleavages other than the first. The effect is shown not only by the percentage of cleavage, but also in its regularity.

When tests were made to establish cleavage-toxicity, the concentration required was found to be 1 : 600 or $N/39$ −. Agglutination of the sperm is good in 1 : 5,000 $NiCl_2$ when one drop of the egg sea-water is added to 7.5 c.c. of thick sperm suspension made up in the membrane-inhibiting concentration. The sperm are active in such a solution even before the addition of the egg sea-water.

9. *Cadmium Chloride.*—Cadmium differs from the other metals studied in only one respect and there the difference is quantitative and not qualitative. When eggs are placed in the membrane-inhibiting concentration, which is 1 : 1,333 + or $N/122.1$, and after various exposure times, are transferred to normal sea-water and inseminated, there is a very marked poison effect after two minutes exposure. Not only does two minutes exposure cause very irregular development, even at the first cleavage, but it tends to inhibit the elevation of membranes. After four minutes, there were 98 per cent. membranes formed and 12 per cent. were narrow. After one hour, all were narrow. When eggs were exposed to the solution for one hour and then inseminated, the first cleavage gave 16 per cent. regular forms and 42 per cent. irregular.

The effect of this concentration on the sperm is very marked. They agglutinate readily with egg sea-water and can be activated by one drop of $N/500$ NaOH or egg sea-water though they are

quiescent in the solution alone. The percentage of membranes was raised from 0 per cent. to 84 per cent. by increasing the sperm concentration at insemination from one drop of 1 : 25 to 12 drops of the same suspension. The higher sperm concentration of this test resulted in much polyspermy.

It would be expected that by increasing the concentration of the $CdCl_2$ in solution, a cleavage-toxic point could be determined. I was successful in this; such a concentration is 1 : 250, or N 23 —.

10. *Cobalt Chloride.*—When a series of tests was made to establish the concentration of $CoCl_2$ necessary to inhibit the elevation of membranes, and the concentration necessary to inhibit completely the formation of the first cleavage in eggs which have been inseminated in normal sea-water and transferred to the solution five minutes after the insemination, it was found that both are much higher that in the other metals w th which I worked. The membrane-inhibitory point was found to be 1 : 1373, or $N/89.245$, while cleavage-toxicity occurred at 1 : 100 or $N/6.5$. As was the case with Cadmium, though not to such an extent, the toxicity of even the membrane-inhibiting concentration on eggs which had been inseminated in normal sea-water and trans'erred to it, was very marked.

Agglutination is good in the solution. The membrane-inhibiting concentration makes the sperm sluggish but this harmful effect is corrected by the addition, of the NaOH or a drop of the concentrated egg sea-water.

11. *Mercuric Chloride.*—As has already been stated above, there is one metallic salt which is irregular in its behavior. This is Mercuric Chloride. All the other salts with which it has been possible to work have a double effect on the eggs of *Arbacia*. The first effect is at the weaker concentration and is on the phenomenon of membrane elevation; the second effect is constantly at a greater concentration and is dependent on the time of exposure of the gametes for the intensity of its action. It modifies materially the viability of the eggs and I have termed it "cleavage-toxicity." The tests made with $HgCl_2$ show that there was no concentration which would inhibit the elevation of membranes and allow cleavage if eggs were placed in it five minutes after they were inseminated in normal sea-water.

TABLE VIa.

Concentration of HgCl₂ in Sea-water.	Per Cent. Membranes.	Per Cent. 1st Cleavage.
1 : 1,000,000.................	100	16
1 : 800,000..........	100	12
1 : 600,000.....	100	0
1 : 400,000....	100	0
1 : 300,000.................	100	0
1 : 200,000.................. ...	100	0
1 : 100,000.....	52	0
1 : 75,000.....	48	0
Inseminated control in sea-water.....	100	100
Uninseminated control in sea-water. .	0	0

Test with HgCl₂. July 11, 1922. 11 : 20 A.M. Eggs and sperm fresh. Insemination 1 : 25 : 7.5. Eggs ♀ C. Temp. 20° C. Concentrations greater than 1 : 300,000 showed cytolysis in 20 minutes.

For example, an examination of Table VIa will show that at 1 : 600,000, HgCl₂ is toxic to cleavage, while there is 100 per cent. elevation of membranes which are wide and to all appearances absolutely normal. In those concentrations in which cleavage does not follow membrane elevation, cytolysis commences within an hour for those eggs with membranes. Eggs without membranes are more resistant. It appears from Table VIb that at 1 : 7,500 HgCl₂, membranes are not raised. At first glance it would be thought that this is a direct inversion of the ratio found to hold for the other metals where membrane-inhibition occurs at a lower concentration than cleavage-toxicity. An examination of the effect of such a solution on the sperm shows that they are instantly paralyzed by it and that this paralysis is not reversible, *i.e.*, neither NaOH nor concentrated egg sea-water will activate the sperm in it. This immediately differentiates such membrane-inhibition from that described for the other metals.

Lillie ('21) notes that the action of HgCl₂ is very different from that of CuCl₂. He notes that "the initial stages are relatively little affected, . . . the susceptibility increases as fertilization progresses" (page 140). He also notes that "mercury also suppresses the movements of the spermatozoa at great dilution." According to his data (Table VII.), a concentration of 1 : 625,000 completely inhibits cleavage while 1 : 15,625 prevents membrane elevation. He states further, that at the latter concentration, sperm are "paralyzed instantly." My observations are in accord with the above, save that it was found

necessary to use a concentration of 1 : 7,500 HgCl₂ to instantly paralyze sperm and consequently to completely inhibit membrane elevation (Table VI*b*). Lillie considered that the action of

TABLE VI*b*.

Concentration of HgCl₂ in Sea-water.	Per Cent. Membranes.	Per Cent. 1st Cleavage.
1 : 75,000	86	0
1 : 50,000	70	0
1 : 25,000	40	0
1 : 20,000	30	0
1 : 15,000	20	0
1 : 10,000	4	0
1 : 7,500	0	0
Inseminated control in sea-water	98	99
Uninseminated control in sea-water	0	0

Test of HgCl₂. July 21, 1922. 3 : 04 P.M. Temp. 26° C. Eggs ♀ C. Eggs and sperm fresh. Insem. 1 : 25 : 7.5.

mercury on membrane-inhibition was like that of copper but at a much higher concentration. I would prefer to consider that the action of HgCl₂ is a poisoning of the sperm, and not the type of inhibition that is present in copper-inhibition.

In most of the metallic salt solutions, the effect is to make the membranes narrow as the concentrations approach the strength necessary for membrane-inhibition; HgCl₂ has exactly the opposite effect. When eggs are inseminated in solutions which do not paralyze the sperm, membranes elevate immediately and such membranes are extremely wide. The solutions alone will produce membranes on eggs in concentrations greater than 1 : 150,000 as has also been observed by Lillie ('21). These membranes form much later than those produced by insemination so that it is easy to differentiate between the two. Eggs with membranes cytolyze very rapidly when left in HgCl₂ solution.

It would thus appear that, in comparing the action of the Mercuric salt with those of the metals already described, the only great difference is in the failure of the HgCl₂ to show any concentration which prevents the action between viable egg and sperm and consequently the initial response of the cortex of the egg.

b. Comparative Tests.

The preceding part is devoted to individual tests on the behavior of various salts. These tests were made with separate

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lots of eggs, under different conditions, and are therefore not as suitable for comparison as they would be had they all been done simultaneously with one set of eggs. Three sets of experiments were therefore set up for the purpose of comparison.

The experiment which appears in Table VIIa, was made to

TABLE VIIa.

Time of Transfer after Insemination.	Percentage First Cleavage. Chlorides of:									
	Au[1]	Cu[1]	Zn	La	Al	Pt	Pb	Ni	Cd	Co
Immediate..	0	0	0	0	0	0	0	0	0	0
30 sec......	29	38	87	98	94	96	80	70	80	80
1 min....	46	64	96	98	92	95	84	78	87	92
2 "	80	89	98	100	97	96	92	90	98	94
4 "	84	98	100	100	100	98	98	92	100	98
8 "	84	99	100	100	100	99	100	98	100	99
15 "	92	99	100	100	100	100	100	100	100	99
30 "	96	99	100	100	100	100	100	100	100	99
Inseminated control in sea-water...............	98	99	100							

Comparative test with membrane inhibitory concentrations. July 15, 1922 Experiment 10 : 15 A.M. Temperature 21° C. Sperm and eggs fresh. Insemination 1 : 25 : 7.5. Membranes formed well, save in immediate inseminations.

[1] In the above experiment, the results for Au and Cu are interpolated.

show the effect of the various solutions on the viability of the same lot of eggs under the same conditions. In each case the concentration used is that of membrane-inhibition. In this table it will be noted that only the figures for the first cleavage are given. In all save the immediate inseminations, membranes were present on a high percentage of the eggs. The table, therefore, emphasizes the great difference between the inhibition of initial and subsequent events, and shows the great similarity of the action of all of the salts. In this table, the figures for AuCl₃ and CuCl₂ are interpolated. It was found, however, that both of these membrane-inhibiting concentrations proved more toxic to cleavage than those of the other salts. I have not used HgCl₂ in this test as it shows no membrane-inhibiting point.

The experiment summarized in Table VIIb was performed to determine the time when superficial cytolysis first begins to appear in uninseminated eggs placed in membrane-inhibiting

concentrations of each of the metallic salts. Readings were made at the end of fifteen minutes, one half hour, and at half-hour intervals thereafter for four and one-half hours. In this

TABLE VII*b*.

Metallic Salt.	Superficial Cytolysis Begins. Hours.
AuCl$_3$	$\frac{1}{4}$
HgCl$_2$	$\frac{1}{2}$
CuCl$_2$	1
ZnCl$_2$	1$\frac{1}{2}$
LaCl$_3$	2
AlCl$_3$	2
PtCl$_4$	3$\frac{1}{2}$
PbCl$_2$	3$\frac{1}{2}$
NiCl$_2$	3$\frac{1}{2}$
CdCl$_2$	3$\frac{1}{2}$
CoCl$_2$	4
Control in sea-water	None in 4$\frac{1}{2}$ hours.

Test of time of cytolysis in membrane-inhibitory concentrations. Eggs, 1 drop to 7.5 c.c. solution. 10 : 45 A.M. 7/26/22. Eggs fresh, 2 ♀s. Temp. 22.5° C.

experiment, one drop of the uninseminated eggs was placed in 7.5 c.c. of the membrane-inhibiting concentration of each of the metallic salts. The container, which was a Syracuse watch-glass was then rotated to insure a thorough mixing of the gametes and the solution.

TABLE VII*c*.

Metallic Salt.	Action Alone.	1 Drop N/500 NaOH Added.	1 Drop Concentrated Egg Sea-water Added.
AuCl$_3$	Active.	Very active.	Very active. Agglut.
CuCl$_2$	"		" " "
ZnCl$_2$	"	Paralyzed.	" " "
LaCl$_3$	Inactive. Clumped.	Clumped. Some activity.	" " "
AlCl$_3$	Active. Clumped.	Clumped. Very active.	" " "
PtCl$_4$	Active.	Very active.	" " "
PbCl$_2$	Active. Some clumping.	" "	" " "
NiCl$_2$	Active.	" "	" " "
CdCl$_2$	Quiescent.	" "	" " "
CoCl$_2$	Sluggish.	Fairly active.	" " "
HgCl$_2$	Paralyzed.	No change.	No change.

Effect of membrane-inhibitory concentrations on the sperm. July 18, '22. Sperm suspension made with dry sperm in 7.5 c.c. of the solution. Sperm from two males.

The experiment in VII*c* was with the membrane-inhibiting concentration of each of the metals and its effect on the sperm.

In this series, a sperm suspension was made by adding a number of drops of the dry sperm to 7.5 c.c. of the membrane-inhibitory concentration of the metallic salt, and the effect of the solution on the behavior of the sperm was noted. A drop of NaOH (N/500) was then added to one preparation and its effect noted. To another preparation, a drop of concentrated egg sea-water was added. The results may be seen in Tab'e VIIc.

c. Summary.

In summarizing the results of the foregoing experiments, it may be generally stated, that, with the exception of $HgCl_2$, all of the metals studied have three effects on the fertilization period of *Arbacia*. The first and second occur at a greater dilution than the third and involve membrane elevation. When eggs are inseminated directly in certain solutions, they give no cortical response, *i.e.*, the effect is immediate and prevents any reaction between the gametes themselves; if, however, eggs are placed in this same solution after cortical discharge has begun but before it is complete, a "narrow membrane" results which may be attributed to an incomplete cortical response. The third involves the subsequent events and requires a higher concentration of the metallic salt. It seems from the data to be cumulative in its action. When the eggs are placed in the solutions five minutes after the insemination in normal sea-water, the time factor amounts to approximately forty minutes, as first cleavage is used as an indicator. If the eggs are transferred at a later time, first cleavage may be only partially inhibited but there will be no second cleavage. That is to say, the time required for the salt solution to produce its toxic effect is, in these experiments, about thirty-five or forty minutes. Table VIII gives a comparison of the concentrations necessary to produce these effects. The order of toxicity of the metallic salts will be seen to be the same for both membrane effects as well as subsequent events. The general results are in accord with those noted by Lillie in his work on copper. Mercury, as has been observed above, is an exception to this general rule.

In each case, as can be seen by reëxamination of Table VIIc, sperm may be caused to agglutinate in the membrane-inhibitory

TABLE VIII.

Metallic Salt.	Membrane-inhibition.		Cleavage-toxicity.	
	Parts.	Normality.	Parts.	Normality.
AuCl$_3$.....	1 : 1,500,000	N/151,800	1 : 375,000	N/37,912.5
CuCl$_2$.....	1 : 500,000	N/ 42,030	1 : 62,500	N/ 8,405
ZnCl$_2$.....	1 : 150,000	N/ 10,215		
LaCl$_3$.....	1 : 50,000	N/ 4,090		
AlCl$_3$.....	1 : 40,000	N/ 1,780	1 : 10,000	N/ 445
PtCl$_4$.....	1 : 11,111	N/ 936	1 : 1,945	N/ 163.87 —
PbCl$_2$.....	1 : 4,000	N/ 554		
NiCl$_2$.....	1 : 5,000	N/ 324	1 : 600	N/ 39 —
CdCl$_2$.....	1 : 1,333.33	N/ 122.1	1 : 250	N/ 23 —
CoCl$_2$.....	1 : 1,373	N/ 89.245	1 : 100	N/ 6.5
HgCl$_2$.....			1 : 600,000	N/81,000

The above table shows the series of metals as established for both membrane and cleavage inhibition in *Arbacia*. With the exception of HgCl$_2$ they are in the order of increasing concentration.

concentrations by the addition of egg sea-water, thus demonstrating that the action is not primarily on the sperm before their interaction with the female gametes.

V. DISCUSSION.

There are three periods definable in *Arbacia* from the moment of insemination up to the first cleavage. The first involves the behavior of the spermatozoön alone and includes its activation, migration to the egg, and its agglutination to the egg. The second involves the reaction of both egg and sperm, and lasts from the end of the latent period to the completion of the cortical activation of the egg. It can, of course, be subdivided. The third is concerned with the rotation of the sperm head within the egg cytoplasm, and the migration of the egg nucleus. In other words, it is the preparation for the union of the germ nuclei and the preparation for cleavage. A latent period comes between the time of the agglutination of the sperm to the egg and the initial events of cortical activation, as described by Lillie ('21). It belongs to the first period described rather than to the second.

The differentiation between the first and second periods is very markedly emphasized by the foregoing experiments. When eggs are placed in solutions indicated in the text as membrane-inhibitory, and immediately inseminated, the sperm are seen to

agglutinate to the egg. This inhibition, therefore, very evidently occurs between the periods of agglutination and of activation, or during the latent period. It may be said that the egg is held at the latent period by the solution. As soon as the agglutination is seen to occur, there is a block interposed, and the reaction ceases. At the time when this block is first interposed, there is no great harm done to the eggs. If they are removed to sea-water and inseminated, the sperm penetrate and cortical activation ensues. This seems to show that the action involved affects only the very external parts of the gametes and is easily removed. After an exposure of slightly longer duration, the viability of the eggs becomes poorer, but this I would consider to be due to the penetration of the salt into both the cortex, and, later, the central part of the egg and consequently, to a very different mechanism from that which prevents the elevation of the membrane.

There are a number of physical and chemical phenomena which may be responsible for this inhibition of membrane formation. The theory of chemical combination was suggested by Lillie. He remarks ('21) the similarity of the effective concentrations found to hold in the "cleavage toxicity" of his work and in the enzyme poisoning discussed below. The physical action may be of two kinds. It may be an adsorption phenomenon resulting in either a change of the electrical charge on the membrane which is present before fertilization or an adsorption by molecular groups. Heesch ('21) finds that the charge on cell membranes of *Lycopodium* spores, leucocytes, and yeast cells can be reversed by the use of $La(NO_3)_3$ solutions. Whether this action is the same as that in membrane-inhibition or not, is an open question. Membrane-inhibition might be due to a change of charge of the egg membrane, or to an adsorption of the metallic ions by some of the complex molecular groups in the egg protoplasm. Were these groups those of the activable substance, this adsorption would explain the partial activation described above. The first of these would involve an electrical action while the latter would result in a chemical inactivation though not a chemical reaction.

Lillie ('21) noted the close similarity between the concentra-

tions of $CuCl_2$ required for cleavage-toxicity and that found by v. Euler and Swanberg ('20) to be toxic to the action of saccharase. McGuigan ('04), in an extensive set of tests of the effect of the metallic salt solutions on the action of diastase, determined the concentrations which inhibit its action for a series of metals. Olsson ('21) also worked on the effects of the various salts of silver and copper on the action of amylase. v. Euler and Swanberg ('21—II) found that the action on saccharase by organic substances was not effected by the temperature. They conclude that there is a binding of the enzyme through the aldehyde group. McGuigan compares the action of the salts to the solution tensions of their ions, though he finds certain irregularities in the series. His results for diastase and those of v. Euler and Swanberg for saccharase are in very close agreement.

The data concerning membrane-inhibition in *Arbacia* do not agree with the results noted by the above authors for enzyme-poisoning. While chemical activity could explain the inhibition effect satisfactorily, it would be difficult to conceive an immediate correction of this, effect if the inhibiting factor were removed. As has already been noted, when the eggs are removed to seawater from a solution in which inhibition is complete, if the exposure has not been too long, there is an immediate recovery and the eggs will produce membranes on insemination in the normal manner.

The present paper emphasizes the distinction to be made between the initial events in fertilization as they are indicated by the cortical activation, and the subsequent events which lead to the period of cleavage.

VI. CONCLUSIONS.

1. The chlorides of the following heavy metals inhibit membrane formation in *Arbacia* in the following order and concentrations;—Au—$N/151,800$, Cu—$N/42,030$, Zn—$N/10,215$, La—$N/4,090$, Al—$N/1,780$, Pt—$N/936$, Pb—$N/554$, Ni—$N/324$, Cd—$N/122.1$, Co—$N/89.2$. The effect of these concentrations upon the cortical response of the egg is immediate. Eggs inseminated $1 : 25 : 7.5$ in these solutions immediately become bombarded by many sperm, no one of which is able to activate the cortex of the egg at the above concentration.

2. In contrast to the above, but showing the same general order, are the concentrations of the same metallic salts which are toxic to cleavage. These salts, with their toxic concentrations are;—Au—$N/75,825$, Cu—$N/8,405$, Al—$N/445$, Pt—$N/163.87$, Ni—$N/39$ −, Cd—$N/23$ −, Co—$N/6.5$. There was so much precipitation in the case of Zn, La, and Pb, that the concentration of these salts, toxic to cleavage could not be determined.

Mercuric chloride proved toxic to cleavage at $N/81,000$, while seeming to favor membrane elevation. At a concentration inhibiting membrane elevation, sperm were paralyzed immediately.

These concentrations vary slightly for different batches of eggs and show the influence of a time factor, thus giving an interesting contrast to membrane-inhibition, the concentrations of which are more constant and immediate in their action. These concentrations are greater in every case except Hg, than the membrane-inhibiting concentration of the same metal. Cleavage toxicity is a progressive, or a cumulative action.

3. These solutions are not immediately harmful to the gametes, but prolonged exposure is injurious to the eggs as can be shown by two sets of experiments.

·(a) When the female gametes are exposed to the solutions for various periods of time and are then inseminated in fresh sea-water, the viability always stands in inverse ratio to the length of exposure.

(b) When the inseminated eggs are transferred to the solutions at intervals after insemination in normal sea-water, the length of exposure to the solution bears an inverse ratio to the viability of the eggs.

It will be seen in both (a) and (b) that the time factor is the important one. The action here is again cumulative.

4. With the exception of mercuric chloride, any harmful effect of these solutions on the sperm is corrected by the addition of a drop of egg sea-water.

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THE BEHAVIOR OF CALCIUM PHOSPHATE AND CAL-CIUM CARBONATE (BONE SALTS) PRECIPITATED IN VARIOUS MEDIA, WITH APPLICATIONS TO BONE FORMATION.

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WITH THIRTY-EIGHT FIGURES IN THREE PLATES.

CONTENTS.

INTRODUCTION.

There are at present three main theories presented in explanation of the manner in which the matrix of bone becomes impregnated with the two inorganic salts, calcium phosphate and calcium car-bonate. These views may be briefly summarized as follows:

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1. That the salts are deposited in the matrix by a precipitation *in situ* from the interaction of soluble salts in the blood and the tissue.

2. That the salts are excreted, or secreted, either with the matrix, or into the matrix, by the bone cells.

3. That a complex combination salt known as calcium carbono-phosphate, carried in solution in the blood, is thrown out of solution in the bony matrix by a change in the carbon dioxide content of the tissue, and after precipitation is finally converted into the two components, calcium carbonate and neutral calcium phosphate, in the exact proportions found in bone.

The weight of evidence is at present in favor of the third view, especially supported by the work of Barillé and of Wells, but it seems to me that fully conclusive proof that this is the process has not yet been brought forward.

The precipitation theory, however, offers a very simple and plausible explanation, and so it was determined to investigate the behavior of bone salts on precipitation and see if any light could be shed on the actual manner of impregnation of the bony matrix.

An exact knowledge of the way in which the bone salts are added would be a great aid, indeed, to our understanding of the growth of bone, repair of fractures, changes in rickets and osteomalacia, and other kindred conditions. It would also throw light on the formation of calcareous patches in scar tissue and in arteriosclerosis, for Wells has shown that calcification and ossification are quite similar processes, and the same two calcium salts are present in both cases in exactly the same proportions.

This research work, as finally carried out, involved the microscopic study of the reactions whereby calcium carbonate and calcium phosphate were precipitated, both in separate solutions and also in the same solution, first in various aqueous media and then in certain colloidal ones. This was followed by a thorough examination of the unchanged matrix of many different bones in the mouse, the frog, the guinea pig, the dog, and human fœtuses of various ages.

"For experimental investigation of the artificial process will

furnish the best clue to a precise and certain knowledge of the natural one, by showing more clearly how much is due to physical agency "—Rainey.

PART 1. MICROSCOPIC STUDY OF PRECIPITATION.

TECHNIQUE.

For simple examination of a reaction, a glass slide measuring 38 x 75 mm. was taken and on it were outlined two squares with sides of 20 mm. painted in melted paraffin, so that the wax formed the sides of a shallow cell. Into a cell were placed a small amount of the two reacting solutions to give the desired precipitate, and then a cover glass 22 mm. square was dropped on, supported by the wax cell wall. If it was desired to keep the specimen, melted wax was then painted all around the edges, and overlapping on to the cover glass. The wax was then coated with thick shellac, which in its turn overlapped slightly on to the glass. The cell was thus permanently sealed. Two cells could be made on one slide and the same reaction could be compared in distilled water and in a colloidal solution side by side.

It was found desirable to examine not only the precipitate formed immediately at the line of contact of the two reacting solutions, but also what was formed later, in more remote parts of the solution, during diffusion, and as the concentration of the dissolved salts became less and less with increasing precipitation. To do this required larger cells, and it was desirable to have a fairly uniform thickness of cell, which was accomplished as follows:

On a glass slide 38 x 75 mm. in size two thin parallel strips of mica dipped in melted paraffin were so placed as to support the long edges of a glass cover slip measuring 24 x 50 mm., which was placed upon them. These long edges were now thoroughly painted over with melted paraffin, which on hardening cemented the cover slip to the slide. A cell measuring from 0.1 mm. to 0.2 mm. deep was thus made, still open at both ends. By means of a fine glass pipette one of the reacting solutions, $M/10$ calcium chloride, was now introduced at one end until the cell was nearly two thirds filled. This end was then carefully dried and sealed with paraffin.

At the still open end was now introduced the other solution—for instance, $M/4$ sodium carbonate, or $M/4$ triple sodium phosphate—and the last border of the cell was then sealed and the slide labeled. All borders were then painted with thick shellac, which was allowed to slightly overlap the wax on to the glass.

To obtain a series of cells of uniform depth, a sheet of mica four inches by two inches in size was split until the required thickness was obtained, equal to that of a number two cover glass. It was cut across with scissors into two-inch strips about two millimeters wide. One sheet thus prepared will cut into approximately forty or fifty strips.

Cells filled with warm solutions sometimes showed broken cover glasses on cooling. Also in cells completely filled with solutions a slight amount of expansion due to change of temperature was sufficient to cause leakage by loosening the wax from the slide. And so to provide for permanency of the specimens all cells were so filled as to leave an air space to provide for contraction or expansion without undue strain, and were filled with solutions at room temperature, 20°–21° C.

Pure distilled water was used in all experiments, all solutions were filtered to make them perfectly clear, and chemically pure reagents were employed in every case.

REACTION IN AQUEOUS SOLUTION.

Precipitation of Calcium Phosphate.

Calcium phosphate was obtained by the interaction of $M/10$ solution of calcium chloride and $M/4$ solution of triple sodium phosphate in water. This strength of reagent gave plenty of precipitate, without it being so dense as to interfere with proper microscopic examination. As the two solutions were of different concentration, there was a different rate of diffusion on the two sides of the line of contact, with corresponding differences in rate and amount of precipitation.

Immediately that contact occurs between the two solutions a milky cloud appears, which grows rapidly in size. For a moment, although the cloud is visible to the naked eye, the solution seen microscopically is still clear, but an instant later it becomes vio-

lently agitated, and the agitation becomes quickly defined as being caused by very minute particles, which are in very rapid motion, both oscillatory and translatory. These particles, appearing only as small dots even under the highest magnification, grow in size, losing their motion as they increase in bulk, until finally they form the fine amorphous granules which gather in clumps, and masses, and fine films, to form the precipitate.

Precipitation of Calcium Carbonate.

Calcium carbonate was precipitated by the interaction of a $M/10$ solution of calcium chloride with a $M/4$ solution of sodium carbonate. The process here resembles that described above for calcium phosphate in the form of myriads of fine particles in rapid Brownian movement. All movement finally ceases as these particles agglutinate into large clumps, and then a remarkable change occurs, for the granular masses just formed fade from the sight, dissolving again into the solution, while scattered here and there larger, rapidly growing particles occur, which become defined as crystals, angular in outline, or as small spherical bodies, known as spherules, which are later converted into crystals as they increase in size (Figs. 1, 2, 3, and 25).

Two or three hours after the formation the precipitate is all crystalline. It is very rare to see a spherule persisting in pure aqueous solutions.

The appearance of precipitates of calcium carbonate in the form of spherules has been frequently noted before. As long ago as 1839 it was described by Link, who, in accordance with this phenomenon, thought that crystals at their first origin were fluid and only later became hard and angular, as solidification occurred.

PRECIPITATION IN COLLOIDAL SOLUTIONS.

The colloids employed were gelatin and egg albumen.

Various concentrations of gelatin were experimented with, ranging from an 8 per cent. solution, which solidified quickly, to a 1 per cent. solution, which remained fluid for some time and then formed a soft jelly. As the various concentrations gave similar results the weaker ones were used as a matter of routine, being easier to handle.

For albuminous solutions Merck's powdered soluble egg albumen was employed, water being added to make the approximate normal proportions given by Lillie for fresh albumen in the hen's egg, namely, albumen 12 per cent., water 88 per cent. by weight.

In 1857 Rainey published the first of a series of papers on the precipitation of carbonate and phosphate of calcium in colloids, using gelatin, albumen, and gum arabic. His best results were obtained with gum arabic, and he pointed out that the solutions of the gum holding the two salts reacting to give the precipitate should be of very different densities, so that diffusion and the consequent reaction would occur slowly and gradually. I have found, however, with albumen and gelatin, that if the reacting salts differ sufficiently in concentration, the concentration of the colloidal solution need not differ in order to secure good reactions. And so in many series of experiments 1 per cent. gelatin was used throughout, although in others this concentration was used only with calcium chloride, and a 4 per cent. solution with sodium carbonate or phosphate. Egg albumen was used always in the same concentration.

Precipitation of Calcium Phosphate in Colloids.

The employment of a colloidal solution seems to have little or no influence on the precipitation of calcium phosphate. The deposit is quite similar to that in aqueous solutions, being fine, granular, and amorphous (Fig. 36), and aggregating to form thin gelatinous veils and clouds. Results in egg albumen were similar to those in gelatin. These findings are in agreement with the work of Rainey, Harting, Biedermann, and others.

Precipitation of Calcium Carbonate in Colloids.

The reaction here is very characteristic. Calcium carbonate formed in the presence of colloids has a tendency to separate out and persist in the form of the spherules mentioned previously, which have been named calcospherites.

The first effect of the colloid is to very much emphasize and prolong the stage in which the particles are small and show active Brownian movement, apparently by preventing their early fusion into larger masses. In a short time, however, larger particles are

seen which grow partly at the expense of the smaller ones, which redissolve as described for aqueous solutions. But there are many small particles which apparently do not dissolve, but pass on directly over to the larger form of deposit, by forming a nucleus for the laying down of additional material from the clear solution.

The precipitate exhibits two separate forms. One of these is distinctly crystalline (Figs. 8, 10, 12, 13) and is found most densely at the primary line of contact of the reacting solutions where deposit was very rapid. Even here the colloid shows its influence, for the crystals are not perfect, but are deformed in various ways, and exhibit rounded angles and suppression of their typical shape.

The second form of deposit is in the shape of very perfect small spheres (Figs. 7–12, 29, 30, 31), which vary greatly in their transparency, markings, and size according to the solution in which they are formed.

Rainey obtained spherules in gum arabic only after the lapse of an hour or more. At first a faint nebulosity was seen, lasting in thin solutions of gum for one hour, in thick solutions for over a week, after which spherules occurred. In my work I have been able to identify these bodies in less than ten minutes, and in some solutions to see them attain a size of 30μ in one hour. They grow rapidly and attain practically their full size in forty-eight hours, ranging then according to the solution in which they lie, from a size of 1μ up to 120μ. In Rainey's case they grew gradually for several months and ranged in size from 2μ to 200μ. I had one single example of a pear-shaped body which measured $120 \mu \times 300 \mu$.

Evidently gum arabic exhibits a more powerful colloidal influence on the precipitate, but gelatin or albumen will give excellent results in a very much shorter time, which is a valuable consideration.

Brownian movement in parts of some slides is still active after the lapse of a year, due to the colloid retaining some of the precipitate in its original, extremely small, granular form, preventing coalescence to form the larger particles seen in other areas where precipitation was denser and more rapid.

The process of coalescence of the small particles can be observed in favorable cases. Very small spherules showing active move-

ment travel about in the solution, colliding with and working past others.   Suddenly two of these, instead of passing, will be drawn together, as though pulled by a strong attractive force, and will merge completely in one large spherule, which in its turn may absorb small ones.   At these moments of coalescence the spherules give the observer the impression of being fluid, flowing together like two drops of water, and lead one to believe that Link was correct in interpreting these small spherules as liquid crystals.   As these bodies become larger they lose their movement, settle down, and become of glassy hardness.   There is also a later growth by addition of material from the surrounding clear solution.

Richards and Archibald have shown that the particles of pre-cipitates at their first appearance seem to have a rounded outline to the eye even under high magnification, but by photographs, how-ever, they have demonstrated that these particles are real crystals of typical shape.   This condition is true for some particles of calcium carbonate formed in aqueous solutions, but in colloids a great deal of the deposit is in the form of particles which are round, and persist in this shape as they grow.   There are other particles also, which clearly are not round, and give the crystalline form of deposit which occurs simultaneously with the spherules.

The spherules formed in gelatin (Figs. 13 and 31) are very perfect, with a smooth surface, and clear, transparent, glassy ap-pearance.   Those formed in albumen (Figs. 26 and 29) often show a slightly irregular periphery and give indications of their internal structure.   They appear to be built up of concentric layers, and often exhibit an amorphous center.   Extending from this center to the periphery is also a faint, but quite distinct, radial striation in certain cases.   These appearances have already been well described and illustrated by Rainey, Harting, and Biedermann.

It ought to be noted here that Harting has been erroneously credited by many writers with the discovery, in 1872, of the calco-spherites formed in colloidal solutions.   Rainey described and gave accurate illustrations of these bodies in 1857, and had observed them as early as 1849, and so deserves the credit of this discovery.

Combined precipitates of calcium carbonate and phosphate (Fig. 36) show each salt acting quite independently, the phosphate occur-

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ring in its usual amorphous form, the carbonate in typical crystals and spherules. Similar conditions were found by Rainey in gum arabic.

The colloids gelatin and albumen were chosen as being artificial media easily prepared which might be looked on as approximately comparable respectively to the bony matrix and to the blood serum or to cellular tissues. Reactions in gelatin might give a clue to those in bone, and similarly reactions in albumen might indicate processes of calcification in other tissues. After studying the reactions in these simple media, they were further modified by the addition of various substances which are either normally or pathologically found in the blood or tissues, to see what effect these special substances would exert.

Further experiments were also carried out to study the effect of the hydrogen-ion concentration of the colloid. To complete this stage of the work reactions were followed in cartilage extract and in blood serum. Then specimens of bone from various animals were examined to see if there was any correlation between the structure of their matrix and the results obtained experimentally in the various media.

The Influence of Other Agents on the Precipitate.

The substance whose influence was to be investigated was added in equal concentration both to the sodium carbonate or phosphate, and to the calcium chloride solutions which were to react to form the precipitate. The new agent was thus balanced so that its action could not be one sided.

The agents used and their concentration in solution were:

$M/10$ sodium chloride, same proportion as in human blood serum.
0.1 per cent. dextrose, same proportion as in human blood serum (Gradwohl).
0.05 per cent. urea, same proportion as in human blood serum.
0.1 per cent. lecithin. Blood serum contains 0.7 per cent. (Wells).
1.0 per cent. acetone. Simulating conditions in acidosis.
0.5 per cent. ethyl alcohol.

The effects produced on the precipitation of calcium carbonate were quite definite for some of these substances, and in some in-

stances very remarkable changes of form resulted. The influence on calcium phosphate was much less striking, as this deposit remained in the same granular, amorphous form in all cases, and the results were mainly a slight difference in the average size of granules, and also differences in the speed and completeness of precipitation similar to those which will be noted in detail below for calcium carbonate, to which latter deposit alone the succeeding paragraphs relate.

Addition of Sodium Chloride.

The main effect noted on adding sodium chloride was the speeding up of the reaction. Brownian movement ceased sooner, crystallization was more rapid, and in most cases the main portion of the precipitate consisted of smaller crystals and spherules, and in the colloids the crystals were of very poor shape.

Addition of Dextrose.

The influence of this compound was slight and seemed to consist mostly in added transparency of the crystals and spherules. There was also slight modification in shape.

Addition of Urea.

Even in aqueous solutions some spherules were formed when urea was present. The effect in colloidal solution (Figs. 30 and 35) was to favor increase in size of the spherules, with a marked tendency toward fusion, so that many double forms and dumbbell-shaped bodies were seen. Urea thus shows an influence quite the opposite of sodium chloride upon the precipitate. It evidently favors growth of this deposit and suggests the idea that the presence of urea in blood and in urine may aid in the deposit of calcium salts in the calcareous incrustation of sclerotic arteries and the growth of renal and vesical calculi.

Addition of Lecithin.

Lecithin, even in small amounts, 0.1 gm. in 100 c.c. water gives a turbid solution even after filtering, but microscopically the solution appears clear, so that it is evidently a colloidal one.

The lecithin has a remarkable influence on the precipitation of

calcium carbonate. Even in aqueous solution (Figs. 4, 5, 6) spherules occur in great numbers, and many of these are of a large size not obtainable in other solutions. There is also a distinct tendency toward fusion of spherules in contact with each other, so that double and multiple forms are numerous. Beautiful rosettes (Fig. 5) were present, due to the adhesion of many oval-shaped bodies.

In gelatin and albumen (Figs. 10 and 11) the same phenomena occurred, producing magnificent rosettes and very large spherules measuring 120 μ in diameter.

Lecithin thus has a very typical colloidal action on the precipitate, favoring the spherical forms of deposit. This compound is found in blood in seven times the strength it was employed here, and is also found in bone and other tissues, so that its action on a precipitation of bone salts, if such occurs, ought to be appreciable. This point will be discussed later.

Addition of Acetone.

The presence of acetone (Fig. 12) hastens the process of precipitation, making it occur very rapidly, and also makes the initial deposit at the moment of contact much denser and more complete. This point is rather suggestive. It leads to the question as to whether or not conditions of acidosis such as accompany serious cases of nephritis and diabetes have an influence favoring the progress of calcification in the arteriosclerosis so often found in these cases.

Addition of Ethyl Alcohol.

The alcohol had no appreciable effect upon precipitation except to add to the optical clearness of some preparations.

THE INFLUENCE OF DIFFERENT IONS.

The calcium carbonate was precipitated in separate cells by the carbonates of sodium (Figs. 1 and 4), potassium (Figs. 2 and 5), and ammonium (Figs. 3 and 6), so that the solutions contained the three last-named ions as the only variable, all other factors being equal. In aqueous solution the crystal form of the calcium carbonate in the three cases was noticeably different. In colloidal

solutions the spherules seen were very different in their appearances in each set of slides.

The most noteworthy change was seen where the ammonium salt was used, the deposit being much coarser, with rougher, more irregular surfaces, and as a rule much greater in size and with more tendency toward fusion and toward clumping into masses.

The whole series for each ion, including those slides with the six accessory agents described above, showed similar tendencies to the plain, unmodified reaction. Each series consisted of 21 slides, consisting of 7 in water, 7 in gelatin, and 7 in albumen.

PRECIPITATION IN BLOOD SERUM.

Clear serum was pipetted off from test tubes of dog's blood allowed to stand for some hours in a refrigerator. The precipitation of calcium phosphate in this serum showed the usual granular amorphous character. Calcium carbonate separated out almost entirely in the form of spherules, many of which were quite large. The process of precipitation in blood serum was very complete, covered practically the whole area of the slide, and seemed to be greater than usual in amount. This appearance suggested the thought that the sodium carbonate and phosphate of the blood aided the reaction, making precipitation greater in amount, but further consideration made this seem unlikely, as the usual amount of carbonate or phosphate was added to the blood, and this alone was sufficient to precipitate more than the total amount of calcium, without the aid of the salts naturally in the blood.

Mixtures of carbonate and phosphate present the usual character, each salt being precipitated in its own peculiar form and being thus easily identified (Fig. 36).

PRECIPITATION IN HYALINE CARTILAGE EXTRACT.

An extract of fresh hyaline articular cartilage was prepared by mashing it up in distilled water, allowing the mixture to stand a short time, and then filtering it and using the clear solution. The same concentrations of salts were used here as in other cases, but the resulting precipitate was very much less in amount. Precipitation occurred only in the area of contact of the solutions, was in

a finely divided state, showed some tendency to form crystals, but mostly was in form of granules and very small spherules. There were no large spherules whatever. The total amount of deposit would lead one to think that very weak solutions had been employed instead of the usual ones.

Hyaline cartilage extract thus seems to decrease the amount of precipitation and to retain what deposit is formed in a very finely divided state. Addition of the various accessory substances used in previous cases had no effect, even lecithin apparently being powerless to produce the large spherules.

These facts may have some significance, for here we have an extract of a tissue which although apparently similar to other cartilage which does ossify, and although situated in continuity with ossified tissue, yet retains a matrix free from a deposit of calcium salts. Its extract forms the most unfavorable medium yet found in which to produce precipitation of calcium salts. Solutions of equal concentration in other media give three to four times the amount of precipitate found here. The experimental facts, it seems, are in complete accord with the nature of the tissue, which is unfavorable to the deposition of calcium salts in it.

INFLUENCE OF HYDROGEN-ION CONCENTRATION.

Loeb's method was employed of treating gelatin to obtain it with a definite hydrogen-ion concentration. To 0.25 gm. gelatin in a test tube were added 20 c.c. of cold acid solution of a definite strength, and this was allowed to stand for 30 minutes, during which time the gelatin swelled remarkably, but did not dissolve. It was then filtered and washed on the filter paper four times with 25 c.c. of cold distilled water at $5°$ C. to remove all free acid.

The gelatin was then transferred to a test tube standing in hot water, where it melted, and the volume was made up to 25 c.c. with hot distilled water, thus giving a one per cent. gelatin solution. Two tubes for each strength of acid were prepared. On cooling the pH of each pair was determined approximately by Clark's colorimetric method, and then one tube of the pair was rendered a $M/10$ calcium chloride solution, and the other one a $M/4$ sodium carbonate solution, and these two solutions were placed as usual in a cell to react.

There was also an alkaline series made similarly to the above. In each series there were ten grades as follows:

Acid series treated with $\dfrac{1}{16}, \dfrac{1}{32}, \dfrac{1}{64} \cdots \dfrac{1}{8192}$ HCl.

Alkaline series treated with $\dfrac{M}{16}, \dfrac{M}{32}, \dfrac{M}{64} \cdots \dfrac{M}{8192}$ NaOH.

The pH of the acid series of washed gelatins in one per cent. solution ranged from 3.2 to 6.0. Untreated gelatin, both in sheets and in powder, which had been used for all the previous experiments was found to possess a pH of 6.0.

The weakest alkaline member of the series registered 6.2 and the fifth member of the series a pH of 9.0, above which no record was kept.

The amount of precipitate visible to the naked eye was less in the acid series than the alkaline one. It was least of all in those specimens where the pH was in the neighborhood of the isoelectric point of gelatin, which occurs in the acid series at 4.7, and which marks the point where the gelatin is most insoluble and most inert. In these slides there was practically no diffusion and spreading of the precipitate, only a narrow band of deposit occurring along the line of contact of the reacting solutions. Such a condition would lead one to infer that the salts in solution are intimately bound to the gelatin.

It was also noted that crystals (Figs. 17, 18, 19) were abundant, and very often large, in the various acid gelatins. Crystals were also present in the weaker alkaline specimens, but in gelatin treated with $M/2048$ NaOH or stronger alkaline solutions (Fig. 23) not a single crystal has yet appeared in a period of six months since the preparation of these slides. Here the precipitate is still in the form of very small spherules, often so densely packed as to prevent Brownian movement, but elsewhere still actively in motion, which has gone on continuously for six months without a change, and gives every indication of continuing indefinitely. The larger of these spherules, approaching one micron in diameter, have a slow movement and so can be well observed, and have given definite proof of what was already suspected from a study of other slides,

namely, that the so-called spherules are in reality flattened discs. During their vibratory movement many of them have been observed to slowly roll over and a narrow, thin edge is presented to the view instead of the circular outline. The appearance reminds one very strongly of a red blood corpuscle.

The largest and clearest spherules (Figs. 19–22) were formed in gelatin whose pH was between 4.7 and 7.5—that is, all in gelatin on the alkaline side of the isoelectric point—but in specimens whose reaction was either weakly acid or weakly alkaline, the neutral point being 7.0. This is the zone in which is obtained the formation of the largest spherules. Untreated gelatin, used for all the previous series of reactions without regard to hydrogen-ion concentration, has a pH 6.0 and so is within this zone. The pH of human blood and many tissues also lies in the alkaline part of this zone.

It is interesting to note that in strong acid members of the series precipitation is rapid, and while spherules may early be present, the deposit in the course of a short time becomes entirely crystalline in structure. In strongly alkaline members precipitation is very complete, but the deposit remains for an indefinitely long time in the form of small, discrete spherules, which do not grow or fuse, and which continue to exhibit Brownian movement. No crystals appear in these latter cases, even after the lapse of many months.

In the intermediate zone of weak acid, neutral, or weak alkaline gelatin both spherules and crystals appear in quantity, and the spherules are very large. In the part of this zone in which the hydrogen-ion concentration of blood and many tissues lies the deposit is mostly in the form of small spherules and the tendency to crystallization is very poor. This is essentially a colloidal phenomenon and seems significant in view of the fact that it has frequently been found that certain drugs in colloidal form have a much better therapeutic effect, have a much less damaging effect on healthy tissues, and are much more pleasant in their administration to the patient. As an example of this, compare the actions of silver nitrate and of argyrol, a colloidal silver vitellin. The colloidal preparation much more nearly approaches the condition of the tissues themselves and the reactions ought to be of a more col-

loidal character, which I have shown is the natural condition with the hydrogen-ion concentration on the basic side of the neutral point. To the acid side of this point reactions tend to the ordinary chemical crystalline variety.

DISINTEGRATION OF SPHERULES.

A most remarkable phenomenon was observed during the last two months in some of the slides, where spherules have been observed in process of disintegration and transformation into crystals. This was first noticed in the acid gelatin series in a slide where the gelatin had a pH of 4.9 and where for the whole of six months appearance had been constant.

This case (Fig. 31) had offered one of the most perfect examples of large spherules, which were of perfect outline, glassy transparency, absolutely clear, and showing no indication at all of their internal structure. These spherules were formed in half an hour and had attained their full growth in less than two days, and for six months presented the same appearance, with no indications of a change. It happened at the end of this period that they were not examined for about one month, when it was discovered that some of them (Figs. 19 and 33) had disappeared, others were disintegrating, and all still present were opaque, while in this area which had not previously shown a single crystal there was now a number of large crystals.

Other slides were examined for similar conditions without avail, but a month later several of them showed signs of a change, and by examination of these at intervals a complete series of stages in the transformation has been obtained and a complete description of it can now be given.

The first sign of a change is in the occurrence of a very fine opaque central spot of a bluish tint (Figs. 20, 21, 24a, and 32) in the otherwise clear spherule. This area increases a little in extent, and the next stage then is ushered in by the appearance around this center (Fig. 24b) of a faint nebulous haze, which becomes more opaque and finally seems to consist of a fine radial striation (Fig. 24c) which extends farther and farther, until it reaches the periphery of the spherule, which is now an entirely opaque white

body. At this stage some spherules are uniformly opaque (Fig. 24d), others show a marked radial striation (Fig. 24e).

The final step in the change now occurs with a very gradual disappearance of the spherule, apparently by dissolving into the surrounding solution. It decreases in diameter and also in thickness until the remnant, diminished very much in size, appears like a nebulous ghost of the former spherule and eventually fades completely from view. During the solution the opaque center of many spherules seems to dissolve early (Figs. 24f and 32), converting the body into a ring.

In those cases where the spherule is uniformly opaque (Figs. 24d and f) solution is very uniform at the surface, the contour of the spherule remaining perfect while the size contracts. Where there is radial striation, however (Fig. 24e and g), the periphery dissolves unevenly, giving the spherule a rough, rather ragged surface.

Coincident with the beginning of the process of dissolving of the spherules, crystals occur in these areas which formerly were entirely composed of spherules, and which for several months' duration had not shown a single crystal. These crystals grow steadily both in size and in number (Figs. 19, 20, 21), while the spherules disappear. The passage of the material from the spherule into solution is evidently very transitory, it coming out again immediately to be deposited in the crystal.

What is the interpretation of this phenomenon? It seems to me that there is a very evident one, and that this is the same process very much delayed in starting, and very much protracted in its action, which occurs rapidly in ordinary aqueous solutions. It will be remembered that in precipitating calcium carbonate in pure water it is at first in a granular, amorphous form, which later redissolves and comes out of solution a second time as spherules and crystals, which grow rapidly, the spherules all being converted into crystals during growth.

Again in colloidal solutions the same process happens, but is delayed somewhat, taking hours or days instead of minutes. In such cases an area of spherules seen one evening after a precipitation, when examined next morning is found to consist of crystals.

Apparently in some of these cases at least, as Brownian movement ceases and the particles grow in size, the spherule is transformed directly into the crystal.

In the degeneration of the large spherule it seems to me we have a similar phenomenon to that of the other cases. The large spherules could correspond to the amorphous particles of the aqueous solution, fused into a large mass, the redissolving occurring very late to form the final crystal. There is evidently a very strong directive force toward the formation of crystals even in the colloids, and though interfered with and hindered by them for a long period, it nevertheless conquers. What it is that initiates the change after the deposit has remained stationary for many months is problematical. There was no infection in the gelatin, no change in its appearance, or any other fact to give a clue.

Some slides in other series in both gelatin and albumen also show evidence of some change in the spherules, which in view of the above facts may be expected to progress farther.

As far as I know this remarkable transformation of the precipitate has never been described before. The further investigation of this phenomenon is outside of my province and belongs to the field of physics and chemistry, where it will provide some very interesting problems which I hope some competent investigator will undertake to solve.

A second method of disintegration (Fig. 6) has also been observed in a lecithin solution, where some large spherules with very marked striation lost this striation after the lapse of several months and became dully opaque. Certain clear radii then appeared along which cleavage occurred, splitting the spherule into sectors which finally separated, giving triangular crystals.

NUMBER OF FORMS ASSUMED BY PRECIPITATE.

It is to be remarked that in no slide was it possible, after Brownian movement ceased, to obtain only a single form of deposit. There were always two, and in many cases several forms occurring, and this applies equally to pure aqueous and to colloidal solutions. This multiplicity of form in the precipitate has also been noted by Biedermann and is due doubtless to differences in the relative con-

centration of the reacting salts and in the diffusion currents in different parts of the solution. Side by side may be found an area of crystals and of perfect spherules, or both forms may be seen mixed in one area.

In passing from the sodium carbonate end of a cell across the precipitate to the calcium chloride end there is often seen a marked change in shape of the deposit, forms present to one side of the line of contact of the two solutions not being present on the other. Spherules may occur all to the one side, but follow no rule as to which they select. Transition forms occur from one area to another. Orde found similar conditions on sectioning plugs of coagulated albumen with which he had sealed the ends of capillary tubes containing solutions which gave precipitates in the plugs when the sealed ends of the tubes were immersed in certain other solutions.

The multitude of forms exhibited in the precipitate of calcium carbonate in the slides seemed remarkable, but on referring to Goldschmidt's "Atlas der Krystallformen," I found illustrations for 2,544 modifications of the crystal form of calcite which have already been observed. Many of the changes are slight, but all are quite evidently different, and among them may be recognized all the types of crystal form I have obtained. Spherules and their variations were not shown.

PART 2. EXAMINATION OF BONE.

TECHNIQUE AND MATERIAL.

The ordinary method of examining bone by means of very thin ground sections, or by sections cut from decalcified bone, are useless to give the structure of the matrix. A method just published by Bast, however, for the study of bone cells *in situ*, without cutting sections, is equally applicable for the examination of the matrix and was here employed.

Bast worked with the parietal and ethmoid bones of mice and young rats, rabbits, cats and dogs, the nasal conchæ of these animals, pieces of the ethmoid in man, and other bones thin enough to be transparent. These were mounted whole. The specimens were fixed in 95 per cent. alcohol, washed in water, stained for

8 hours or more in diluted aqueous solution of Gentian violet, dehydrated in alcohol, cleared in benzol, and mounted in balsam.

To stain the bone cells only, the periosteum was left on the specimens, and this was later removed by dissection under a binocular microscope while the bones were in benzol. In this way the matrix remained clear, while if the periosteum was removed previous to staining, the matrix stained also.

This method was used by me, but as the matrix was the main object of study, most of my preparations were not stained, but were mounted for observation clear, in three different ways: first, without fixation, in a shallow cell filled with normal saline; second, in a shallow cell of glycerin; and third, fixed in 95 per cent. alcohol, then absolute alcohol, cleared in benzol, and mounted in balsam.

A cover glass to which some of the precipitate of calcium carbonate was adherent in the form of both spherules and crystals was passed through all the processes necessary to staining above and mounted in balsam. There was no change to be seen in the precipitate, so it can be inferred that calcium salts in the bony matrix, if in this form, would not be altered by any of the processes.

The specimens of bone used were parietal and ethmoidal bones and nasal conchæ of the mouse, frontoparietal bones of the frog, and splinters and thin sections in various planes from the femur, tibia, humerus, radius, ulna and scapula, of frog, mouse, guinea pig, dog, and human fœtus. The frog and mouse were young, so that their bones were in process of growth, and if there is any visible difference between new formed and old matrix it should be evident here. Thin pieces of the long bones cut with a sharp knife to take in areas of the total thickness of the shaft, or to show transition from the bony shaft to the cartilaginous epiphysis, were taken, splinters were clipped off long bones, small pieces were crushed, and all these were mounted as described above. The whole of a concha, scapula or parietal, if small, was mounted as one specimen.

In all cases the periosteum was carefully peeled off and was mounted alongside of the bone so that examination of it for newly formed matrix could also be made.

RESULTS OF EXAMINATION OF BONE.

Both bright and dark field illumination were employed and
specimens of bone, when thin, were quite transparent and easily
examined. In bone that is well formed, whether from adult, or
young individual, or fœtus, the appearances are similar. The bone
cells and all their processes in the canaliculi are easily followed and
appear as Bast has described them. The matrix round about them
is homogeneous, transparent, and almost glassy in appearance, and
gives no indication (Fig. 37) of being formed of separate parti-
cles. If it was originally formed in such a manner, there has been
complete fusion, so that there is no evidence of a precipitation. In
fact, the whole appearance bears out the statements of text-books
of histology that, although two thirds of the bony matrix is com-
posed of inorganic salts, they are so intimately blended with the
organic material that there is no visible evidence of their presence.

In the case of growing long bones, such as those of the limbs,
sections were taken across the shaft to include the newly formed
growing periphery just under the periosteum, and also sections in
the length of the bone to include the end of the diaphysis, the
epiphyseal plate of cartilage, and the bony epiphysis. These sec-
tions, however, were disappointing, as even at the transitional areas
any tissue that could be identified as bony matrix appeared homo-
geneous, so that no evidence of precipitation could be obtained here.

In the case of the developing diaphysis of the long bones of the
limbs in human fœtuses, however, there were definite, discrete
particles to be seen. In Quain's Anatomy there is a fine de-
scription by Schäfer of the appearances of developing bone with
which my findings entirely agree. Just ahead of the advancing
bone of the diaphysis is an area of change, where the tissue is no
longer cartilage, but is not yet true bone. Here we find definite
fibres (Fig. 38) like those of white fibrous tissue, and known as
Sharpey's fibres, extending from the bony material forward into
the cartilage, and in these fibres in the narrow area immediately in
advance of the true bone are numerous very fine granules which
appear similar to the material of the matrix in well-formed bone
and so are evidently the bone salts. They are crowded in the
fibres just ahead of the actual bone, and in the bone itself we find

the homogeneous appearance due to the fusion of these discrete particles into one mass. Pacchioni has also described the first appearance of lime salts in bone in the form of fine granules which later form a homogeneous mass.

The possible origin of these particles either by precipitation or secretion will be discussed later.

PART 3. DISCUSSION OF RESULTS.

Concerning some of the physical phenomena of the precipitation reactions studied, remarks have been made at appropriate places in the previous pages describing the work and need not be repeated here.

There are a few points to be constantly borne in mind in considering the question of ossification, which have been very clearly demonstrated by Wells. The most important is the fact that there is a very definite and fixed ratio between the amount of calcium carbonate and calcium phosphate deposited in bone. The second point is that this same ratio is equally true in areas of calcification in tissues other than bone. The third fact is that the processes of ossification and of calcification are essentially the same, the kind of matrix or tissue in which the deposit occurs differentiating the two processes.

THEORIES REGARDING DEPOSITION OF CALCIUM SALTS.

The reactions with calcium phosphate show it to be invariably precipitated in a granular form so that if it were visible in bone it should be evident in a finely divided amorphous form. As this salt forms over eighty per cent. of the inorganic material, it ought to be readily demonstrated, but this is not the case, because the matrix is clear and homogeneous, not granular, except at the advancing edge of ossification in developing fœtal bones.

It has been shown that in mixed solutions calcium carbonate is not prevented, by the presence of the phosphate, from coming out in typical spherules or crystals, so that it might be expected that the carbonate would show in one of these forms in bone, in which it forms fifteen per cent. of the salts. This expectation is further strengthened by the fact that typical spherules have been found in the shells of invertebrates, which are mostly calcium carbonate, by

Rainey and by Biedermann. The dentine and enamel of the teeth, Rainey believed he had demonstrated to be composed of spherules, forming in rows and coalescing. Neither in old bone nor in new, nor in a transitional area transforming from cartilage to bone, have I seen either a single crystal or a single spherule.

Previous work has demonstrated that spherules can always be found in calcium carbonate precipitated in colloids. This formation is favored by the occurrence of the reaction gradually and slowly, and also by the presence of lecithin, which is found in both blood and bone, so that all the conditions in the matrix of the bone are favorable to the formation of spherules if precipitation occurs. The hydrogen-ion concentration is also favorable to formation of large spherules. With optimum conditions for their occurrence, and with a complete absence of these bodies, the interpretation is that the salts found in the bony matrix are not deposited in it by simple precipitation from a double decomposition of salts in the blood or the tissue. This is purely negative evidence, which, of course, is not of the same value as positive evidence, but it seems to me forms a very strong point against the simple precipitation theory.

It must be noted, however, that if calcium carbonate is very small in amount compared to the amount of phosphate precipitated that spherules may not be in evidence, but the whole precipitate will be granular, with coarser granules, resembling minute spherules and interpreted as carbonate interspersed among the masses of finer granules formed by the phosphate. It must be emphasized that homogeneous masses such as seen in the bony matrix never occurred in any of my experiments, but precipitates, no matter how long they were kept, remained composed of distinct discrete particles.

Another point against this theory is that it supposes that a soluble salt of calcium circulates in the blood to be precipitated as phosphate and carbonate in the bone. With the large amount of sodium carbonate and sodium phosphate in the blood, calcium carbonate and phosphate should be formed at once, here and not in the bone. But calcium is present in the blood in a greater amount than calcium carbonate and calcium phosphate are soluble in water.

And so it might appear that calcium can not be carried in the blood in the form in which it is later deposited in the bone. Pauli and Samec, however, have shown that difficultly soluble salts like calcium carbonate may have the amount of their solubility in water increased to over seven-fold in an albuminous solution, and so it is possible that the bone salts, formed by interaction of the blood salts and calcium absorbed into the blood as calcium chloride, might still be carried in solution in the blood and deposited in the bone.

This is closely related to the views of Barillé, whose theory is a step in advance of this, and is strongly supported by such work as that done by Wells. According to this view the calcium is in the blood in the form of a double salt formed here, tribasic calcium carbonophosphate, $P_2O_8Ca_2H_2 : 2CO_2(CO_3H)_2Ca$. This is soluble in the blood concentration of carbon dioxide. Infiltrating tissues like the bony matrix where the carbon dioxide content is lowered, this is precipitated and immediately breaks up into calcium carbonate and dicalcium phosphate, which latter salt is converted into calcium phosphate, giving the final proportion found in bone of 15 parts calcium carbonate to 85 parts phosphate. Even by this method of precipitation, however, one might expect to be able to see the salts in the matrix, and not being able to do so argues in favor of their being combined with the matrix. The most important point brought out in this theory is a clear consistent explanation supported by experimental evidence, as to the fixed proportion in the amount of the two calcium salts, the carbonate and phosphate, which is always found in their deposition.

It is interesting to note here the following facts regarding cartilage. Wells has shown that there is an almost specific power in cartilage for the absorption of calcium salts. My work is in agreement with this, for in cartilage extracts there was obtained the least amount of precipitation obtained in any of the experiments, thus showing the power of the substances in cartilage to hold on to the calcium. In spite of this affinity for calcium, however, microscopic evidence of developing ossification in bones preformed in cartilage shows us that the deposition of calcium is never directly in a cartilaginous matrix, but that the cartilage preceding the bone is destroyed and replaced by the organic bony matrix which is of

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a fibrous character, and this then has the calcium salts deposited in it.

The final possible explanation of the deposit of bone salts in the matrix is the secretory one. Wells and others have been forced to the conclusion that there is a selective or specific action of tissues, such as cartilage or membrane about to ossify, by which calcium salts are absorbed or otherwise taken into them, while apparently entirely similar areas of cartilage and membrane do not ossify at that time or at any later time. From this view of selective action it is not a far step to that of secretory action, explaining the ossifying process as a secretion of the calcium salts by the bone forming cells. It is already generally admitted that the matrix is a product of the bone cells, and if this is true, it is quite logical to assume that the calcium salts are taken by the bone cells from the blood and passed on into the matrix.

The difficulty in this theory has been to account for the amount of phosphate in the tissues. The calcium was looked upon as being derived from that carried in the blood, but the phosphate was explained as being due to cellular activities, derived either from the nucleus or from cellular degeneration. Another difficulty here, again, is to explain the fixed ratio of carbonate and phosphate. This, of course, could be looked upon as due to the carbon dioxide content of the tissue, or as due to the activity of the cell, a definite proportion being found in the secretory products here just as in other organs of the body.

It seems to me, however, that the nearest approach to the truth is to be obtained by combining two views. The bone salts, I believe, reach the tissue as the soluble double salt, tribasic calcium carbonophosphate, which by its constitution provides for the tissue both calcium carbonate and calcium phosphate, each salt in the proper proportion found in bone. I do not believe, however, that deposit here is by precipitation, for in the bone, owing to the colloidal matrix, the hydrogen-ion concentration, and the presence of lecithin, all conditions are most favorable to a visible and characteristic precipitate, showing granules and spherules, but there is no visible evidence whatever of these bodies. The only evidence in favor of precipitation consists of the following two sets of facts:

First, Schäfer and Pacchioni have described the first appearance of bone salts in developing bone in the form of minute granules which quickly coalesce. This I have confirmed. This evidence could also be interpreted as showing the presence of the salts to be due to secretory activity. Secondly, Olivier in a tumor of the breast and Pettit in a tumor of the maxilla and also in a renal cyst saw and made illustrations of calcospherites. These bodies can be explained only as a precipitation phenomena, but they do not afford sufficient evidence to prove that this is the process whereby the bone salts are deposited. And so these salts, after reaching the bone in correct proportions as the double salt above mentioned, must be deposited in the matrix by some more subtle process than ordinary precipitation.

Here we have recourse to the secretion theory. The bone salts in their proper proportions having been brought to the cell by the blood, are taken by the cell and secreted as an integral part of the matrix in combination with the ossein, the organic base, which it is generally admitted already, is a product of the cells. This will produce a matrix uniform in appearance, with salts invisible, and provides the proper proportion of each salt, as the cell is in its turn provided with the two salts in that exact amount. Histological evidence shows that the fibrous matrix appears first in the newly forming bone and impregnation with the bone salts begins immediately, the salts showing as fine granules at first, and coalescing as their quantity increases, to form a homogeneous matrix.

Reversibility of Calcium Reaction.

There seems to be evidence that this process of secretion is reversible, the bone cells being able to take up the calcium salts again. Wells and others have shown that calcium is removed from the bones in cases where there is a great demand for it in the body, as in pregnancy, or where it is being steadily lost by the body, for instance by way of a pancreatic fistula. Also, the osteoclasts responsible for the normal resorption of bone are supposed by some authors to be only osteoblasts which have changed their function. And further, where a graft of either living or dead bone, or a bone plate and bone screws are used to repair an injury, a process known

as creeping replacement occurs whereby this material is gradually absorbed and replaced by new bone. It has been shown by Gallie and Robertson that it is the osteoblasts which invade the graft, gradually absorb the old matrix and bone salts immediately surrounding them, and then replace this by newly formed substance.

If the bone salts were laid down by precipitation of tribasic calcium carbonophosphate due to a change of the carbon dioxide content, it seems to me it would be impossible for this precipitate to be removed again as easily as Wells states it is, in response to the body's need for calcium. Would pregnancy or a pancreatic fistula so change the carbon dioxide content of the bone, as to make possible a reversal of the conditions that brought about the precipitation, and so either provide for increased solubility of the two salts or else make possible the recombination of the carbonate and phosphate of calcium into the double salt, with its consequent solution and carrying off in the blood?

It seems to me that the carbon dioxide content in the bone will not vary enough to permit of such a reversal, as to raise it would indicate a high activity in a tissue whose metabolism is low. This low-grade activity is given as a reason for obtaining the precipitate.

The taking of the calcium salts out of the bone probably depends on some balance that has to be maintained between the blood, the osteoblast, and the bony matrix. If the blood is rich in calcium, the cell can take it and pass it on into the matrix. If the blood is poor in calcium, the cell to maintain its relation in balance to the blood takes from the matrix to add to the blood. This is an instance comparable to the relations between the blood and its dextrose, and the liver cell and its stored glycogen, to maintain a certain carbohydrate balance.

This view also follows logically upon that of Wells, who regards the bony matrix as a great reserve store of alkaline bases for the body, where the calcium salts are in a state of flux, being constantly added to or drawn upon according to the needs of the body. Changes in the bones are much greater in extent and more rapid in their occurrence than is generally believed. It seems to me that this idea of the reversibility of the direction in which calcium salts

pass through the tissues is a most valuable one for the proper comprehension of some of the processes of growth, repair of fractures, and changes going on in rarefaction of bony tissues.

SUMMARY.

1. Calcium phosphate precipitated in water or in colloidal solutions is constantly granular and amorphous in character, and apparently uninfluenced by the nature of the solution.

2. Calcium carbonate precipitated in water shows a great diversity of crystalline form; in colloidal solutions it shows two main forms, an irregular crystalline one and a spherical.

3. Mixtures by simultaneous precipitation of both salts in the same solution show each salt separating out independently and the part of the deposit formed by each can be easily identified.

4. Spherule formation by calcium carbonate is a typical reaction in colloidal solutions.

5. Spherules and crystals are influenced in shape, size, number, and internal structure by a variety of substances found normally or pathologically in the blood, most notably by lecithin which favors the formation of large spherules, and by acetone which increases the rapidity and extent of precipitation.

6. The character of the deposit of calcium carbonate is influenced by the hydrogen-ion concentration of the colloidal solution, being most crystalline in acid media, all in the form of spherules in strongly alkaline media, and mixed in form in solutions neutral or nearly neutral.

7. Large spherules, after persisting for months may undergo a sort of degenerative process, with change of internal structure, after which they dissolve and the material forming them is laid down in a crystalline form. As far as I know this phenomenon has never been previously described.

8. In fresh bone of various animals examined in various ways there is no visible microscopic evidence of the bone salts, although they form two thirds of the mass of the matrix. The inference is that the bone salts are not deposited in the matrix by simple precipitation, for the conditions are such that, if precipitated, granules, spherules, and crystals should be visible.

9. In the rapidly developing fœtal skeleton the first appearance of bone in the matrix is in the form of fine granules or globules which quickly fuse or coalesce to form a homogeneous mass. This might be interpreted according to the bias of the observer as supporting evidence either of precipitation or of secretion of the salts into the matrix.

10. The view advanced by Barillé and supported by Wells's work, that calcium is carried in the blood as tribasic calcium carbonophosphate, is probably correct, as it furnishes the bone salts in the proper proportion, but their view of its deposit in the matrix as a precipitate due to change in concentration of carbon dioxide does not appear correct in view of the fact that no precipitate of the bone salts is visible.

11. The theory that the salts furnished by the blood are taken by the bone cells and secreted by them along with the matrix seems reasonable in view of the condition found in the matrix.

12. The action of the osteoblasts seems to be reversible, they being able to absorb or take up the calcium salts again out of the matrix.

In concluding this paper I wish to acknowledge my indebtedness to others by thanking Professor J. Playfair McMurrich for special apparatus and for valuable references, also the following for providing material for investigation: Professor W. H. Piersol, Dr. R. D. Defries, Dr. Banting, Dr. Simpson, Dr. Dafoe, and Dr. Low.

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EXPLANATION OF PLATES.

PLATE 1.

All figures are from camera lucida tracings and are of equal magnification, × 200, so that the relative size of precipitated particles in each case can be compared. Only forms which were common, and appeared in quantity have been shown.

All illustrations are to show forms assumed by calcium carbonate precipitated from various solutions by the reagents listed below.

FIG. 1. Crystals of calcium carbonate precipitated by sodium carbonate in distilled water.

FIG. 2. Crystals of calcium carbonate precipitated by ammonium carbonate in distilled water.

FIG. 3. Crystals of calcium carbonate precipitated by potassium carbonate in distilled water.

FIG. 4. Deposit of calcium carbonate precipitated by sodium carbonate in distilled water containing o.1 per cent. lecithin. Note small spherules, and small crystals, a tendency towards clumping, a very large spherule and a large rosette.

FIG. 5. Deposit of calcium carbonate precipitated by ammonium carbonate in distilled water containing o.1 per cent. lecithin. Spherules of various sizes, a marked tendency to fusion, and formation of large irregular masses are to be noted.

FIG. 6. Deposit of calcium carbonate precipitated by potassium carbonate in distilled water containing o.1 per cent. lecithin. Note a tendency to clumping, various sizes of spherules with some double forms. The last three forms indicate one process of disintegration of a spherule, by the occurrence of radial lines along which it splits into sectors, which thus become triangular crystals.

FIG. 7. Deposit of calcium carbonate precipitated by sodium carbonate in a solution of egg albumen. Practically the whole precipitate was in form of spherules.

FIG. 8. Deposit of calcium carbonate precipitated by ammonium carbonate in a solution of egg albumen. Spherules of varying size are seen, also some crystals.

FIG. 9. Deposit of calcium carbonate precipitated by potassium carbonate in a solution of egg albumen. Many spherules are seen, and a tendency towards fusion is noted.

FIG. 10. Deposit of calcium carbonate precipitated by sodium carbonate in a solution of egg albumen containing o.1 per cent. lecithin. Note the markedly increased size of spherules and crystals and the tendency to fusion.

FIG. 11. Deposit of calcium carbonate precipitated by ammonium carbonate in a solution of egg albumen containing o.1 per cent. lecithin. Note the increased size of particles, and their tendency to fuse.

FIG. 12. Deposit of calcium carbonate precipitated by potassium carbonate in a solution of egg albumen containing 1 per cent. of acetone. Crystals and spherules both occur. One crystal is seen fused with a spherule.

FIG. 13. Deposit of calcium carbonate by interaction of calcium chloride in a 1 per cent. gelatin solution with sodium carbonate in a 4 per cent. gelatin.

Fields are shown by various sized spherules, of crystals, and of spherules and crystals mixed.

FIG. 14. Deposit of calcium carbonate by interaction of calcium chloride in 1 per cent. gelatin solution with ammonium carbonate in 4 per cent. gelatin solution. Spherules of various sizes are seen.

FIG. 15. Deposit of calcium carbonate by interaction of calcium chloride in 1 per cent. gelatin solution containing o.1 per cent. lecithin, with ammonium carbonate in 4 per cent. gelatin solution containing o.1 per cent. lecithin. Note the increase in size of the spherules over those shown in Fig. 14. Three different degrees of fusion are illustrated in the double forms.

FIG. 16. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of untreated gelatin.

FIG. 17. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $\frac{1}{16}$ HCl solution. Large crystalline precipitate.

FIG. 18. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $\frac{1}{32}$ HCl solution. Large, markedly crystalline deposit.

FIG. 19. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $\frac{1}{512}$ HCl solution. The field shown in this figure was composed for some months altogether of clear spherules and a few dumb-bells. The spherules here illustrated are now undergoing disintegration and in their place are appearing large crystals. Some debris is seen in one corner. One spherule is seen partly from the side, showing its true shape to be a disc.

FIG. 20. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $\frac{1}{2048}$ HCl solution. Disintegrating spherules and new crystals are seen.

FIG. 21. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $M/8192$ NaOH solution. Newly formed crystals and various stages of disintegrating spherules are shown.

FIG. 22. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $M/4096$ NaOH solution.

FIG. 23. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $M/2048$ NaOH solution. Precipitate is all in the form of small spherules which have been in constant Brownian movement for six months.

FIG. 24. A series of spherules showing various stages in the disintegrative changes which occur before they redissolve. a, Spherule with small central opaque area. b, Spherule with opaque radial striation extending from central spot. c, Spherule with further extension of opaque area. d, Spherule almost completely opaque. Narrow, bright, clear periphery. e, Spherule striated right out to periphery. f, Spherule like that in d, dissolving both at periphery and in center in a regular manner. g, Spherule like that in e, dissolving at an irregular rate at the periphery. h, j, Remains of dissolving spherules, and debris of some that have fallen apart.
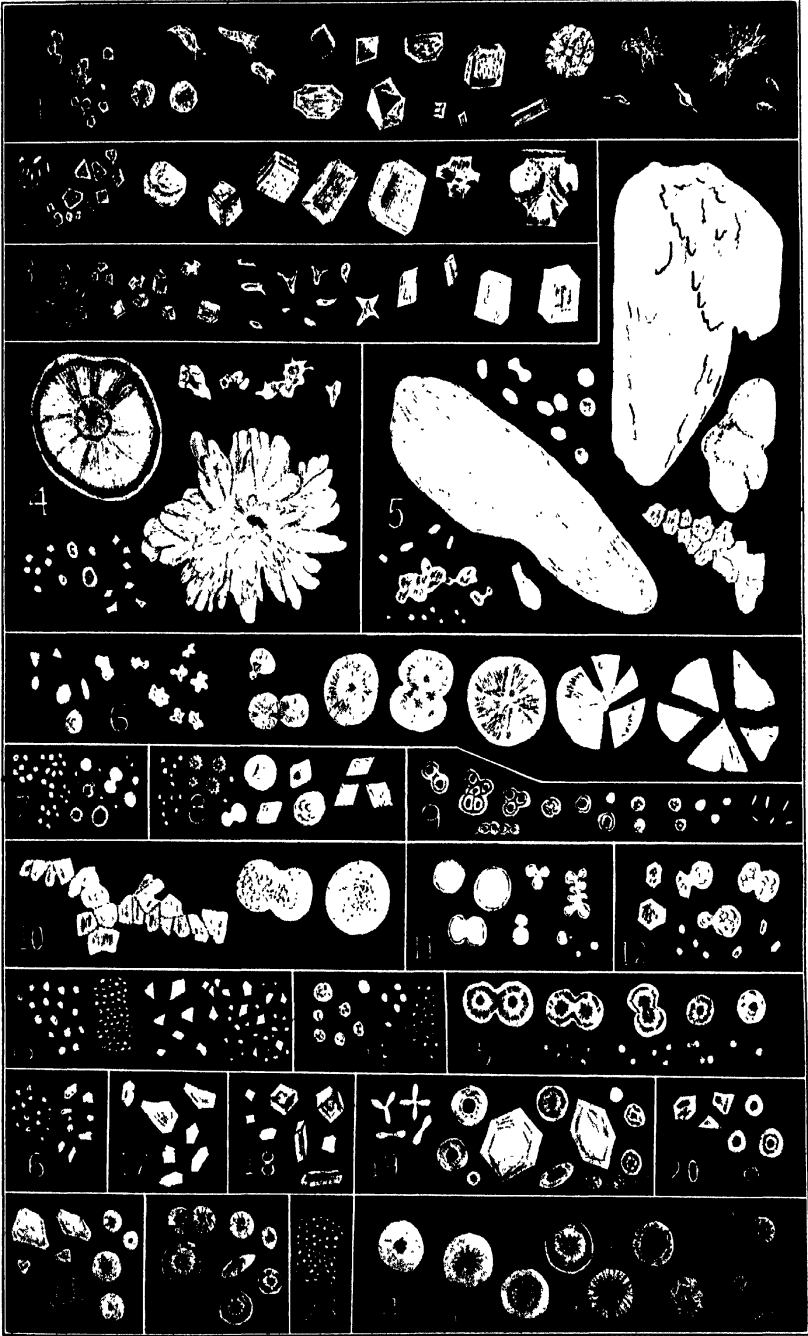
Photomicrographs all taken at a standard magnification, × 200, with dark field condenser in use.

FIG. 25.   Crystals of calcium carbonate precipitated by ammonium carbonate in water.

FIG. 26.   Calcium carbonate precipitated by sodium carbonate in a solution of egg albumen containing $N/10$ sodium chloride.

FIG. 27.   Calcium carbonate precipitated by potassium carbonate in water containing 0.1 per cent. lecithin.   Note one double spherule and three large single spherules of which the two larger show indications of the radial divisions which will result in splitting the spherule into crystals of triangular outline.   Many small crystals and spherules seen.

FIG. 28.   Calcium carbonate precipitated by ammonium carbonate in water containing 0.1 per cent. lecithin.

FIG. 29.   Calcium carbonate precipitated by potassium carbonate in a solution of egg albumen containing 1 per cent. ethyl alcohol.

FIG. 30.   Calcium carbonate precipitated by potassium carbonate in a solution of egg albumen containing 0.05 per cent. urea.

FIG. 31.   Calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with 1/1024 HCl solution.   Clear spherules, some seen from side appearing disc-like.

FIG. 32.   Calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with 1/2048 HCl solution.   Some crystals are present.   Spherules begin to show an opaque central dot, the first indication of a coming disintegration.

FIG. 33.   Calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with 1/512 HCl solution.   This field was formerly all spherules, now some crystals are seen.   Spherules are far advanced in disintegrative process, are completely opaque, and are dissolving, some appearing only as ghosts of their former selves.

FIG. 34.   Calcium carbonate precipitated by ammonium carbonate in 1 per cent. gelatin solution containing 0.05 per cent. urea.   Rings and striations seen in spherules.

FIG. 35.   Calcium carbonate precipitated by sodium carbonate in a 1 per cent. gelatin solution containing 0.05 per cent. urea.   Field of very small mixed crystals and spherules.

FIG. 36.   Combined precipitate of calcium phosphate and calcium carbonate by action of sodium phosphate and sodium carbonate in dog's blood serum. The calcium carbonate shows as seven spherules.   The calcium phosphate shows as a cloud of very fine amorphous granules, which is the form in which it precipitated in all experiments.
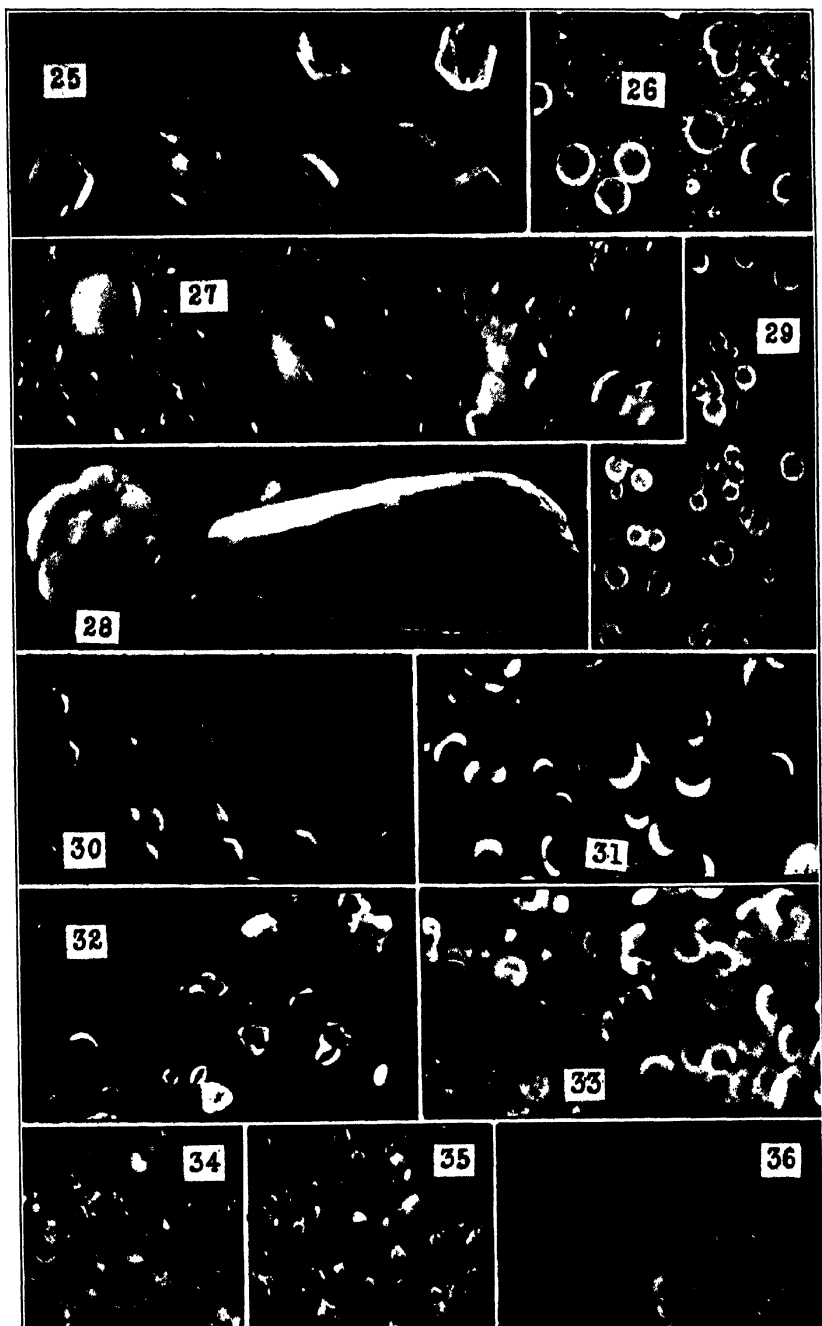
FIG. 37. Photomicrograph of a small piece of bone from the growing femur of a mouse. The bone cells show as small, oval, opaque bodies in the specimen. No discrete particles can be seen in the matrix. Magnification, × 200.

FIG. 38. Outline sketch made with camera lucida, of the growing bony end of the diaphysis of the humerus of a six months human foetus. Four zones can be distinguished. *A,* fully formed bone spicules (shown as solid black, marrow spaces white, bone and marrow cells not indicated) with calcium salts fused. *B,* osteogenic fibres, impregnated with calcium salts in form of minute granules. *C,* newly forming osteogenic fibres, not yet impregnated with bone salts, and cartilage showing changes preparatory to bone formation. *D,* unaltered cartilage. Magnification, × 300.
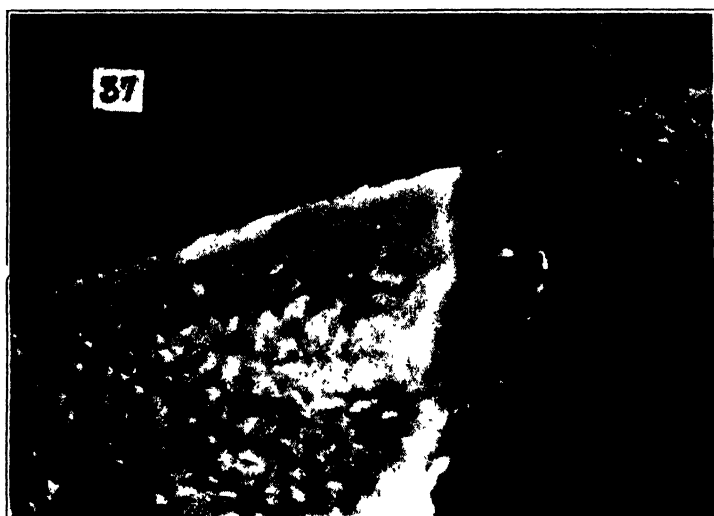
BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

Editorial Staff

Volume XLV.

Contents of Volume XLV

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BIOLOGICAL BULLETIN

THE MARINE BIOLOGICAL LABORATORY.

TWENTY-FIFTH REPORT, FOR THE YEAR 1922, THIRTY-FIFTH YEAR.

I. TRUSTEES.

*Deceased.

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TO SERVE UNTIL 1925.

C. R. CRANE, New York City, *President of the Corporation.*

I. F. LEWIS, University of Virginia.

R. S. LILLIE, Nela Research Laboratory.

E. P. LYON, University of Minnesota.

C. E. McCLUNG, University of Pennsylvania.

T. H. MORGAN, Columbia University.

D. H. TENNENT, Bryn Mawr College.

E. B. WILSON, Columbia University.

TO SERVE UNTIL 1924.

H. H. DONALDSON, The Wistar Institute of Anatomy and Biology.

W. E. GARREY, Tulane University.

CASWELL GRAVE, Washington University.

M. J. GREENMAN, The Wistar Institute of Anatomy and Biology.

*GEORGE LEFEVRE, University of Missouri, *Secretary of the Board.*

A. P. MATHEWS, The University of Cincinnati.

G. H. PARKER, Harvard University.

C. R. STOCKARD, Cornell University Medical College.

TO SERVE UNTIL 1923.

H. C. BUMPUS, Brown University.

R. A. HARPER, Columbia University.

W. A. LOCY, Northwestern University.

JACQUES LOEB, The Rockefeller Institute for Medical Research.

GEORGE T. MOORE, Missouri Botanical Garden, St. Louis.

L. L. NUNN, Telluride, Colorado.

W. J. V. OSTERHOUT, Harvard University.

WILLIAM M. WHEELER, Bussey Institution, Harvard University.

II. ACT OF INCORPORATION.

No. 3170

COMMONWEALTH OF MASSACHUSETTS.

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintain-

*Deceased.

ing a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand, Eight Hundred and Eighty-Eight.

[SEAL] HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY.

I. The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 12 o'clock noon, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk, who shall be, *ex officio*, members of the Board of Trustees, and Trustees as hereinafter provided. At the annual meeting to be held in 1897, not more than twenty-four Trustees shall be chosen, who shall be divided into four classes, to serve one, two, three, and four years, respectively, and thereafter not more than eight Trustees shall be chosen annually for the term of four years. These officers shall hold their respective offices until others are chosen and qualified in their stead. The Director and Assistant Director, who shall be chosen by the Trustees, shall also be Trustees, *ex officio*.

II. Special meetings of the members may be called by the Trustees

to be held in Boston or in Woods Hole at such time and place as may be designated.

III. The Clerk shall give notice of meetings of the members by publication in some daily newspaper published in Boston at least fifteen days before such meeting, and in case of a special meeting the notice shall state the purpose for which it is called.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting; they shall elect one of their number President and may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient.

VII. The President shall annually appoint two Trustees, who shall constitute a committee of finance, to examine from time to time the books and accounts of the Treasurer, and to audit his accounts at the close of the year. No investments of the funds of the Corporation shall be made by the Treasurer except approved by the finance committee in writing.

VIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be given to the Boston Society of Natural History, or some similar public institution, on such terms as may then be agreed upon.

IX. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

X. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

IV. TREASURER'S REPORT.[1]

Harvey S. Chase & Company,

Certified Public Accountants, 84 State Street, Boston.

January 22, 1923.

MR. D. BLAKELY HOAR,
   161 Devonshire Street,
   Boston.

Dear Sir: We have completed our audit of the accounts of the Marine Biological Laboratory for the year ended December 31, 1922, as kept both at your office in Boston and at Woods Hole, and report thereon in the accompanying exhibits and schedules:

Exhibit A—Balance-Sheet as of December 31, 1922.

Exhibit B—Income-and-Expense for the Year ended December 31, 1922.

Schedule I—Investments (Book Values).

Schedule II—Cash Receipts and Disbursements on Account of Funds.

Schedule III—Land, Buildings, and Equipment.

Schedule IV—Supply Department Income-and-Expense Account for the Year ended December 31, 1922.

We certify that, subject to the comments herewith, the balance-sheet and income-and-expense statement shown in Exhibits A and B are in accordance with the books and correct, to the best of our knowledge and belief.

Very respectfully,

HARVEY S. CHASE & COMPANY,
Certified Public Accountants.

[1] Only a part of the audit is included in the Treasurer's Report. The complete audit is on file at the Laboratory and may be examined by any member.

MARINE BIOLOGICAL LABORATORY BALANCE SHEET, DECEMBER 31, 1922.

Assets.

Cash:

In bank...............................	$ 1,592.05	
Petty cash fund...........................	200.00	$ 1,792.05
Accounts receivable.......................................		14,535.47
Inventories:		
Supply department........................	23,960.63	
BIOLOGICAL BULLETIN......................	3,329.25	27,289.88
Investments:		
Securities—Schedule I......................	12,151.12	
Less Loan...............................	1,100.00	
	11,051.12	
Cash—Schedule II........................	516.64	11,567.76
Stock in General Biological Supply House, Inc...		12,700.00
Gansett property account.......................	18,832.22	
Less mortgage............................	10,782.01	8,050.21
Educational Plant—Schedule III:		
Land...................................	95,856.14	
Buildings...............................	209,183.50	
Equipment...............................	99,430.61	
	404,470.25	
Less Reserve for Depreciation...............	51,050.52	353,419.73
Items in suspense:		
First payment on purchase of Fish Estate, Woods Hole...................................	5,000.00	
Expense for surveying and testing ground in re proposed extension of laboratory building.....	418.49	
Sundry items............................	32.16	5,450.65
Prepaid insurance..		1,304.85
		$436,110.60

Liabilities.

Accounts payable..	$ 2,611.66
Notes payable—Falmouth National Bank......................	10,000.00
Accrued charges (estimated)................................	1,200.00
Items in suspense...	59.30
Trust funds..	11,567.76
	$ 25,438.72

Balancing Account:

Balance, January 1, 1922....................	$399,777.13	
Add:		
Balance of income account for year.........	5,894.75	
Special donation received from C. R. Crane for first payment on purchase of Fish Estate.................................	5,000.00	410,671.88
		$436,110.60

Marine Biological Laboratory, Income & Expenses for Year Ended December 31, 1922.

	Expenses	Income	Loss	Gain
Administration expenses......	$ 9,570.85		$ 9,570.85	
Bar Neck Property expense...	318.00		318.00	
Biological Bulletin and annual dues................	4,265.50	$ 3,653.31	612.19	
Biological Bulletin, adjustment of expenses for 1921...	200.21		200.21	
Carpenter department.......	958.18	1.55	956.63	
Chemical department........	2,012.79		2,012.79	
Dormitories...............	2,384.01	2,660.78		$ 276.77
Instruction................	6,866.46	9,390.00		2,523.54
Interest on notes payable.....	700.66		700.66	
Janitor's house expense......	7.84		7.84	
Library department........	1,860.22		1,860.22	
Maintenance, buildings and grounds.................	6,640.42		6,640.42	
Mess.....................	24,328.81	26,184.05		1,855.24
New laboratory............	4,276.30		4,276.30	
Newman cottage............	80.13	150.00		69.87
Pumping station............	587.39		587.39	
Research department........	2,817.16	6,425.00		3,607.84
Sundry expense and income ..	804.11	10,853.61		10,049.50
Supply department (See Schedule IV)..................	44,004.65	49,368.07		5,363.42
Truck....................	783.14		783.14	
Total current expenses...	$113,466.83		$28,526.64	
Total current income........	108,686.37	$108,686.37	23,746.18	$23,746.18
Excess of expenses.......	4,780.46		4,780.46	
Reserve for depreciation......	8,980.35			
Bad accounts written off....	365.97			
	$14,126.78			

Donations for expenses:
Friendship Fund
Inc.... $20,000.00
Others..... 21.53   20,021.53

Balance to balancing
account............ $ 5,894.75

MARINE BIOLOGICAL LABORATORY INVESTMENTS, DECEMBER 31, 1922.

Reserve Fund.

Cash on hand......................................	$ 388.41	
* $3,000.00 American Telephone & Telegraph Company, 4's	2,921.25	
500.00 Western Telephone & Telegraph Company, 5's..	496.88	
* 6 shares American Smelting & Refining Company, Preferred......................................	732.00	
8 shares General Electric Company..................	907.25	
4 shares General Electric Company Special (par $10.00) received as a stock dividend.		
14 shares United Shoe Machinery Corporation, Preferred..	393.75	
5 shares Massachusetts Gas Companies, Preferred.......	444.63	
	$6,284.17	
Items marked * are held as collateral on loan of......	1,100.00	$5,184.17

Library Fund.

$300.00 U. S. Liberty Loan, First 4 1/4's..............	300.00	
4/5 of $1,000.00 American Telephone and Telegraph Company, 4's....................................	779.00	
3 shares American Telephone & Telegraph Company......	362.38	
3 shares General Electric Company....................	346.80	
1 1/2 shares General Electric Company Special (par $10.00) received as stock dividend.		
5 shares United Shoe Machinery Corporation, Preferred..	140.63	
3 shares Massachusetts Gas Companies, Preferred.......	269.38	
*1 share American Smelting & Refining Company, Preferred......................................	122.00	
	$2,320.19	
Less overdraft of cash..............................	120.88	
		2,199.31

Lucretia Crocker Fund.

Cash on hand......................................	249.11	
$300.00 U. S. Liberty Loan, 1st 4 1/4's.................	300.00	
1/5 of $1,000.00 American Telephone & Telegraph Company, 4's...	194.75	
18 shares Vermont & Massachusetts Railroad Company....	2,416.50	
3 shares General Electric Company...................	349.55	
1 1/2 shares General Electric Company Special (par $10.00) received as stock dividend.	.	
1 share Boston Elevated Second Preferred.............	133.00	
1 share American Telephone & Telegraph Company......	120.79	
4 shares Boston Consolidated Gas Company, Preferred...	420.58	4,184.28
Total, Exhibit A..		$11,567.76

V. REPORT OF THE LIBRARIAN.

The growth of the Library continues steadily. The number of books added during the year was 473. Of these eighty volumes were received by purchase, 199 by binding of periodicals, 157 were gifts, and 21 were additions to the permanent loan from the American Museum of Natural History. Our valuable collection of reprints has been increased by the addition of 434 pamphlets.

The number of periodicals currently received was 229; of which 87 are received by subscription, 59 by exchange for the Biological Bulletin, 69 were gifts, and 14 were duplicates lent to us by the American Museum of Natural History. The total contents of the Library at the end of the year is 11,136 volumes and 9,393 pamphlets.

Two of the gifts received during the year are especially noteworthy. The first to be mentioned is the gift from Mrs. George Peirce of books selected from the working library of her husband, a distinguished physiologist and chemist, whose herioc death in 1919 at the height of a productive career of research has retarded the advance of biochemistry in America. This is a collection of thirty volumes, all recent books of importance, and is a very valuable addition to our library.

The second gift of special note is a complete set of the cards of the Concilium Bibliographicum. This is given by the Library of Carleton College through the good offices of Dr. Donnell B. Young of our Instructing Staff. These cards will be of very great use in the bibliographic research that is a prominent part of the work of investigators every summer. But before they can be used these cards must be filed in a suitable cabinet. Until a cabinet and room for it can be provided, the cards can only be stored.

Another notable gift is a collection of thirteen volumes of their publications given by Messrs. P. Blakiston's Son & Company through their representative, Mr. Horace G. White. These books, with one exception, are all published in 1919, or later, and all are desirable additions to the Library.

Dr. Christine Ladd-Franklin has presented to the Library the Concise Oxford Dictionary, edition of 1911, and a copy of Woodworth's Psychology, 1921. From Dr. R. P. Bigelow have been received several volumes, including the U. S. Entomological Commission Reports, Volumes 2 and 3, 1878–82.

Through the kindness of their authors we have received the following books:

B. M. Patton, "Laboratory Directions in Embryology," 5 volumes.

E. G. Conklin, "Direction of Human Evolution."

G. H. Parker, "Smell, Taste, and Allied Senses in Vertebrates."

W. C. Curtis, "Science and Human Affairs."

L. L. Woodruff, "Foundations of Biology."

B. H. Grave, "Birds of Wyoming."

W. A. Collier, "Einführung in die Variationsstatistik."

H. C. van der Heyde, "Physiology, Digestion, Respiration, and Excretion in the Echinoderms."

J. F. Nonides, "Herencia Mendeliana."

Libbie H. Hyman, "Laboratory Manual of Comparative Vertebrate Anatomy."

C. T. Brues, "Insects and Human Welfare."

A. H. Church, "Thallassophyta and Sub-aërial Transmigration."

When the present officers took over the administration of the Library, there was found a large accumulation of printed matter, mostly unbound, that was thought to be duplicates. During the early part of the year covered by this report, the Assistant Librarian spent considerable time sorting this material. Thirty-three volumes were found not to be duplicates and were added to the Library. They are included in the gifts mentioned in the first paragraph of this report. From the sale of 450 duplicates, we received $106.20, which was applied to the purchase of new books. There remain about 150 odd numbers and volumes and 1,000 reprints still to be catalogued.

The Director having authorized an extension of the exchange list, considerable time has been given to selecting and soliciting new exchanges with the Biological Bulletin. Nineteen new exchanges have been established, some of them bringing more

than usually desirable publications, and in the following cases this includes back sets as well as current issues:

Hereditas,
Acta Zoologica,
Annalen des Naturhistorischen Museums, Vienna,
Archives Néerlandaises de Physiologie,
Carlsberg Laboratorium, Comptes Rendus,
Skandinavisches Archiv für Physiologie,
Svensk Botanisk Tidskrift, Vol. 1, 1907, to 16, 1922,
Kolloid Zeitschrift,
Revue générale des sciences pures et appliquées.

The completion of our sets of serial publications is one of the important aims of the library administration. It is, therefore, gratifying to report that we have been able to complete the following by purchase:

Allgemeine botanische Zeitschrift, Vols. 1–25,
Botanische Jahrbücher, Vols. 1–56,
Journal of Pharmacology and Experimental Therapeutics,
    Vols. 1–11,
Zeitschrift für Botanik, Vols. 1–13,
Zoological Record, Vols. 31–57.

The dictionary catalogue which was begun in 1920, now contains 11,051 cards, and work has started on the transfer of cards from the old classified catalogue.

The increase in the use of the Library has continued, the circulation during 1922 being about 1,200 items, besides 30 volumes borrowed from other libraries.

We still lack many reprints of work done at Woods Hole, and authors are requested to look over the reprints in the Library and to send us what are lacking. Among the special needs of the Library are a complete set of the *Philosophical Transactions of the Royal Society,* a good encyclopedia, and bibliographical aids, such as the Royal Society Catalogue of Scientific Papers, and several sections of the International Catalogue of Scientific Literature.

ROBERT P. BIGELOW,
Librarian.

VI. THE DIRECTOR'S REPORT.

To the Trustees of the Marine Biological Laboratory.

Gentlemen: I beg to submit herewith a report of the thirty-fifth session of the Marine Biological Laboratory for the year 1922.

The business of the Laboratory has become so complex, especially in the summer, that the executive officers can no longer assume the responsibility of administering it with reference only of major problems to the Board of Trustees once or twice during the year. The Executive Committee of the Board has accordingly been reorganized during the last two years to consist of the Director and Assistant Director *ex officiis* and three members of the Board resident in Woods Hole during the summer, one member being elected each year for a three-year period. The Committee meets weekly during the summer, and at other times of the year when called, and reports its principal activities at the annual meeting of the Board. In this way different members of the Board are successively brought into close contact with detailed problems of administration of the Laboratory. It is felt that this practice is good for the morale of the entire Institution; it is also a great relief to the administrative officers to share their responsibilities at times when decisions have to be made; naturally, also, it makes for greater steadiness and consistency in the conduct of affairs, through the establishment of precedents.

The season of 1922 was very gratifying as to attendance and all other evidences of active interest and wide coöperation in the affairs of the Laboratory.

Attendance.—The attendance of students in courses was 126, of investigators 182, making a total of 308 persons, who represented 104 institutions. These numbers are considerably in excess of any previous attendance, except in the case of students in courses whose numbers are limited by rule. Applications for working places were very considerably greater than could be granted; about 30 students had to be refused places on account of the rule limiting admission to courses, but it was pos-

sible to provide for nearly all applicants for research facilities. There is no doubt that if the present limitations in the Laboratory were removed, that numbers would mount much higher. For an institution devoted primarily to research, as ours is, this cannot be regarded as an end in itself. But thére is no doubt that the present accommodations are inadequate to fulfill the present strictly justifiable needs of American Biology. This becomes more obvious year by year. If we are to continue to justify the status of the Marine Biological Laboratory as the national center of biological research we must accommodate ourselves to the idea of a future considerable increase in attendance. The investigator who desires quiet for his research must use the same rules of protection in the Marine Biological Laboratory that he does in the still larger university communities from which he generally comes.

The number of subscribing and coöperating institutions increased from 52 in 1921 to 61 listed on page 30; there were in addition three scholarship arrangements providing for five students. Of the total amount of $9,687.50 received from these institutions, $3,912.50 went for students' tables and $5,775.00 for research accommodations. In 1921 the receipts from subscribing and coöperating institutions amounted to $8,800.00. As 58 per cent. of the students paid their own fees in 1922 the fear that has been expressed lest subscribing and coöperating institutions should monopolize all the students' places does not appear to be justified. This is borne out in another way by comparing the total of institutions represented, 104, with the number of coöperating and subscribing institutions, 61. The growth in the number of these institutions is an encouraging sign of continued interest which represents the broad basis of support of the Laboratory.

The Report of the Treasurer (see p. 5).—The earned income of the Laboratory increased from $98,292.38 in 1921 to $108,686.-37 in 1922; and the current expenses increased from $109,970.40 in 1921 to $113,466.83. The excess of current expenses over earned income was $11,678.02 in 1921 and only $4,780.46 in 1922. If it were not for the constant need for additions out-

side of current expenses the Laboratory would be approaching a self-supporting basis. The annual subvention of $20,000.00 a year from the Friendship Fund provides both for the excess of current expenses and also for necessary annual improvements and additions. * The net assets of the Laboratory increased from $430,251.28 in 1921 to $436,110.60 in 1922; and the liabilities decreased from $30,474.15 in 1921 to $25,438.72 in 1922.

The increase of income over 1921 amounting to $10,393.99 is due to a relatively slight increase of the receipts of all departments and a large increase in the income of the Supply Department from $42,774.78 in 1921 to $49,368.07 in 1922.

Great encouragement in our plans for the new buildings proposed since 1919 was received during the year from the Rockefeller Foundation, from the Carnegie Corporation, and from Mr. C. R. Crane. Early in January the Board of the Rockefeller Foundation voted to authorize negotiations with the Marine Biological Laboratory on the basis of one-half of the total sum called for in our estimates, viz.: $1,000,000 for completing the Laboratory, library, and auditorium building and providing endowment for its maintenance. This was conditioned upon the total sum being raised, the continuance of the sum of $20,000 annually from the Friendship Fund, and making of satisfactory arrangements for reversion of endowment in case the work of the Laboratory were to be radically changed or abandoned. In February the Trustees of the Carnegie Corporation appropriated the sum of one hundred thousand dollars ($100,000) to the Marine Biological Laboratory for the purpose of creating a fund the income of which shall be used for the maintenance of its buildings and laboratories provided gifts to the total amount of one million dollars ($1,000,000) be secured for the joint purpose of providing for buildings and for their maintenance. Mr. Crane also assured the Director that the maintenance of $20,000 a year from the Friendship Fund would be guaranteed as soon as the other conditions were met. Circumstances during the remainder of the year did not permit an active campaign to secure the additional funds needed though inquiries have been made that should inform us during the session of 1923 just what efforts will be required.

During the summer farther study of the building plans and of equipment was made and incorporated in working drawings and specifications by the architects. Costs of building having increased since the original estimates were prepared in March 1922, the necessity of some curtailment of building plans was considered, and alternative schemes for constructing major parts of the entire plan were also prepared by the architects. The plans and specifications are ready for the builders as soon as the balance of the sum needed to meet the conditions has been provided.

The Laboratory traces its origin directly back to the impetus given the study of Marine Biology by the establishment of the Anderson School of Natural History on Penikese Island near Woods Hole in 1873 by Louis Agassiz. As the summer of 1923 will be the fiftieth anniversary of this epoch-making event in the history of biology in America, it seemed to be an appropriate time for the establishment of a memorial in memory of this service of Agassiz. On recommendation of Dr. Clapp a committee was appointed by the Trustees to consider and recommend plans for the establishment of an Agassiz Memorial. It is hoped that this may take the form of a suitable monument on the site of Agassiz's laboratory.

Towards the end of the year a most important addition to the real estate holdings of the Laboratory was made by the President of the Board, Mr. C. R. Crane, who presented to the Laboratory the property known as the Kidder House at the corner of East and Water Streets immediately adjoining the old buildings of the Laboratory and forming part of the block on which the first buildings of the Laboratory were erected. We owe a debt of gratitude to Mrs. Kidder as well as to Mr. Crane for the acquisition of this land, which she may be said to have held for the Laboratory pending a favorable opportunity for its acquisition. In the Resolution concerning the death of Mr. Camillus G. Kidder, included in this report, reference is made to the interest of the Kidder family in plans for biological work at Woods Hole at a period even ante-dating the establishment of the Marine Biological Laboratory. It is to that interest

that we owe our most important sites, including the land on which the Crane laboratory stands; the present status of the Laboratory is in an important respect the fruition of the far-sightedness of the Kidder family.

The Trustees at their meeting on August 8, 1922, established a class of emeritus trustees to honor Dr. Cornelia M. Clapp who expressed her desire to be relieved from membership on the Board after twenty-two years of service. This action was taken in acknowledgment of the exceptional spirit of devotion displayed toward the laboratory from its foundation to the present time by Miss Clapp and in the confident expectation of her continued interest. Dr. Clapp was accordingly elected Trustee Emeritus at the meeting of the Board in August 1922.

At the meeting of the Corporation August 8, 1922, two new members were elected to the Board of Trustees to fill vacancies in the newly elected class of 1926: Professor Otto Glaser of Amherst College, and Professor F. P. Knowlton of Syracuse University, both old members of the Corporation and regular investigators at the Marine Biological Laboratory.

At the meeting both of the Trustees and also of the Corporation the deaths of three former Trustees of the Laboratory were commemorated in the following memorials:

Resolution on the death of William T. Sedgwick, drawn up by Dr. Cornelia M. Clapp:

William T. Sedgwick, professor of biology and public health at the Massachusetts Institute of Technology, Boston, died on January 25, 1921.

The career of Professor Sedgwick as a teacher is well known. He was a fluent lecturer, and as one of his students has said he could make science popular and could take subjects of popular interest and clothe them in the language of science. Soon after he came to Boston he became consulting biologist of the State Board of Health and after that was identified with the interests of the American Public Health Association. He has been called the "Ambassador of Health." It is, however, as a member of the Board of Trustees of the Marine Biological Laboratory that we remember him today. In 1887 came an awakening of

interest in a project for starting a sea-side laboratory, and his aid was sought by the Woman's Education Association in their efforts in this direction.

It was proposed that there should be a permanent institution, incorporated, and supported by the educational institutions of the country. There is no name more prominent in this movement than that of Professor Sedgwick. He was one of the original seven trustees, served on committees, suggested and helped carry out plans for raising funds, enlisted the aid of benevolent Bostonians, and not least among his activities was the effort to master the practical details concerned with the living conditions in Woods Hole.

He spent the summer of 1888 in this vicinity looking after the interests of the Laboratory. His devotion to the Laboratory during the earliest and most critical period of its history will be remembered and cherished by the friends of the Laboratory. A life-long friend, Dr. E. B. Wilson, has written an appreciation which it is hoped will find a place among the records of the Marine Biological Laboratory.

Resolution on the death of Camillus G. Kidder, drawn up by Dr. E. B. Wilson:

The Trustees of the Marine Biological Laboratory record with deep regret the death of Camillus G. Kidder, a member of this Board since 1897, and always one of its highly valued friends and loyal supporters. Both Mr. Kidder and his brother, Dr. Jerome H. Kidder, were among the earliest of the summer residents at Woods Hole, and both were from the beginning in close touch with the biological work here carried on, through their close friendship with Spencer F. Baird and his associates in the U. S. Fish Commission. From the first Mr. Kidder showed a sympathetic interest in Baird's plans for the larger development of that work. A conspicuous example of this, and one that should have a place in the annals of the Marine Biological Laboratory, was the early purchase by himself and Dr. Kidder, at Baird's suggestion, of the land on which the new laboratory now stands, in order to hold it in friendly hands with a view to the possible later development of Baird's plans. When

the Marine Biological Laboratory was established at Woods Hole in 1888, Mr. Kidder unhesitatingly extended his interest to the new enterprise, becoming a steadfast supporter of its plans for scientific work and for friendly coöperation with the Fish Commission. Years later it was through him and Mrs. Jerome Kidder that Baird's foresight brought fruit through the acquisition by the Marine Biological Laboratory of the Kidder Land, later to become the site of the Crane Laboratory, and the future site, as we hope, of its further extension.

The Trustees here record their appreciation of Mr. Kidder's wise counsel in the conduct of the Laboratory and of the kindly and understanding spirit in which he took part in the deliberations of this Board and from time to time presided over its meetings. We cherish the memory of his sympathetic personality and generous friendship, and we are grateful for the long continued services that he rendered.

Resolution on the death of Alfred G. Mayor, drawn up by Dr. E. G. Conklin:

The death of Dr. A. G. Mayor, Director of the Department of Marine Biology of the Carnegie Institution of Washington at his laboratory at Tortugas, Florida, on June 24 last is ·lamented as a serious loss by the Marine Biological Laboratory. Dr. Mayor was not only a distinguished organizer and director of research in tropical marine biology and the leading American student of Cœlenterata but he was ever the loyal friend of the Marine Biological Laboratory. He carefully planned the work of his Department of the Carnegie Institution so as to supplement and not to duplicate or interfere with the work of this Laboratory, and at a critical period in our history he said that he would gladly abandon his cherished projects if by so doing he could materially aid the Marine Biological Laboratory. He was a useful member of our Board of Trustees and he assisted in the work of our institution not only by his attendance and lectures but also by furnishing facilities and generous assistance for investigations in tropical waters to members of our Staff and Corporation.

It is especially as a helpful, unselfish, and genial friend that

we love to remember him; he was always ready to sacrifice himself for the good of others and it can be truly said that he gave his life not merely to science but also to his fellow scientists.

The Marine Biological Laboratory expresses to his family its deep sympathy in their bereavement and its high appreciation of his work and character.

There is attached as part of this report a list of the Staff and of Investigators and Students for 1922, a tabular view of attendance 1918–1922, lists of subscribing institutions, of the Evening Lectures and of the Members of the Corporation.

I. THE STAFF.

1922.

FRANK R. LILLIE, *Director*, Professor of Embryology, and Chairman of the Department of Zoölogy, The University of Chicago.

GILMAN A. DREW, *Assistant Director*, Marine Biological Laboratory.

ZOÖLOGY.

I. INVESTIGATION.

GARY N. CALKINS, Professor of Protozoölogy, Columbia University.

E. G. CONKLIN, Professor of Zoölogy, Princeton University.

CASWELL GRAVE, Professor of Zoölogy, Washington University.

H. S. JENNINGS, Professor of Zoölogy, Johns Hopkins University.

GEORGE LEFEVRE, Professor of Zoölogy, The University of Missouri.

FRANK R. LILLIE, Professor of Embryology, The University of Chicago.

C. E. McCLUNG, Professor of Zoölogy, University of Pennsylvania.

S. O. MAST, Professor of Zoölogy, Johns Hopkins University.

T. H. MORGAN, Professor of Experimental Zoölogy, Columbia University.

G. H. PARKER, Professor of Zoölogy, Harvard University.

E. B. WILSON, Professor of Zoölogy, Columbia University.

II. INSTRUCTION.

ROBERT H. BOWEN, Instructor in Zoölogy, Columbia University.

EDWARD F. ADOLPH, Instructor in General Physiology, University of Pittsburgh.

J. A. DAWSON, Assistant Professor of Biology, Dalhousie University.

ANN H. MORGAN, Professor of Zoölogy, Mount Holyoke College.

C. L. PARMENTER, Instructor in Zoölogy, University of Pennsylvania.

J. PAUL VISSCHER, Instructor in Zoölogy, Washington University.

BENJAMIN P. YOUNG, Assistant Professor of Zoölogy, Cornell University.

DONNELL B. YOUNG, Assistant Professor of Biology, Carleton College.

PROTOZOÖLOGY.

I. INVESTIGATION.
(See Zoölogy.)

II. INSTRUCTION.

GARY N. CALKINS, Professor of Protozoölogy, Columbia University.

LOUISE H. GREGORY, Assistant Professor of Zoölogy, Columbia University. (Absent on leave.)

FLORENCE DEL. LOWTHER, Instructor in Zoölogy, Barnard College.

EMBRYOLOGY.

I. INVESTIGATION.
(See Zoölogy.)

II. INSTRUCTION.

HUBERT B. GOODRICH, Associate Professor of Zoölogy, Wesleyan University.

BENJAMIN H. GRAVE, Professor of Biology, Wabash College.

ROBERT S. McEWEN, Assistant Professor of Zoölogy, Oberlin College.

HAROLD H. PLOUGH, Associate Professor of Biology, Amherst College.

CHARLES G. ROGERS, Professor of Comparative Physiology, Oberlin College. (Absent on leave.)

ELIZABETH A. SMITH, Assistant Professor of Zoölogy, University of Wisconsin.

PHYSIOLOGY.

I. INVESTIGATION.

HAROLD C. BRADLEY, Professor of Physiological Chemistry, University of Wisconsin.

WALTER E. GARREY, Professor of Physiology, Tulane University.

RALPH S. LILLIE, Biologist, Nela Research Laboratory, Department of Pure Science, Nela Park, Cleveland, Ohio. .

ALBERT P. MATHEWS, Professor of Biochemistry, The University of Cincinnati.

II. INSTRUCTION.

MERKEL H. JACOBS, Assistant Professor of Zoölogy, University of Pennsylvania.

FRANK P. KNOWLTON, Professor of Physiology, Syracuse University.

ALFRED C. REDFIELD, Assistant Professor of Physiology, Harvard
Medical School.
REYNOLD A. SPAETH, Associate in Physiology, School of Hygiene and
Public Health, Johns Hopkins University.

BOTANY.

I. INVESTIGATION.

S. C. BROOKS, Department of Public Health, Washington, D. C.
EDWARD M. EAST, Professor of Experimental Plant Morphology,
Harvard University.
ROBERT A. HARPER, Professor of Botany, Columbia University.
E. NEWTON HARVEY, Assistant Professor of Physiology, Princeton
University.
WINTHROP J. V. OSTERHOUT, Professor of Botany, Harvard University.

II. INSTRUCTION.

IVEY F. LEWIS, Professor of Biology, University of Virginia.
WILLIAM RANDOLPH TAYLOR, Instructor in Botany, University of
Pennsylvania.
WILLIAM H. WESTON, JR., Assistant Professor of Cryptogamic Botany,
Harvard University.

LIBRARY.

ROBERT P. BIGELOW, Librarian and Associate Professor of Zoölogy
and Parasitology, Massachusetts Institute of Technology. Li-
brarian.
PRISCILLA B. MONTGOMERY (Mrs. Thomas H. Montgomery, Jr.),
Assistant Librarian.

CHEMICAL SUPPLIES.*

OLIVER S. STRONG, Associate Professor of Neurology, Columbia
University, New York City, Chemist.

SUPPLY DEPARTMENT.

GEORGE M. GRAY, Curator.
THOMAS M. DOUTHART, Assistant
Curator.
JOHN J. VEEDER, Captain.
E. M. LEWIS, Engineer.

A. W. LEATHERS, Head of Ship-
ping Department.
A. M. HILTON, Collector.
J. MCINNIS, Collector.

F. M. MACNAUGHT, Business Manager.

2. INVESTIGATORS AND STUDENTS, 1922.

INDEPENDENT INVESTIGATORS—Zoölogy.

ADAMS, A. ELIZABETH, Associate Professor of Zoölogy, Mount Holyoke College.

ADDISON, WILLIAM H. F., Professor of Histology and Embryology, University of Pennsylvania.

ADOLPH, EDWARD F., Instructor in Physiology, University of Pittsburgh.

ANDERSON, ERNEST G., Research Associate, Carnegie Institution, Cold Spring Harbor.

BASCOM, KELLOGG F., University of Chicago.

BIGELOW, ROBERT P., Professor of Zoölogy and Parasitology, Massachusetts Institute of Technology.

BISHOP, MABEL, Professor of Zoölogy, Hood College.

BODINE, JOSEPH H., Instructor in Zoölogy, University of Pennsylvania.

BOWEN, ROBERT H., Instructor in Zoölogy, Columbia University.

BRIDGES, CALVIN B., Research Assistant, Carnegie Institution of Washington.

BUDINGTON, ROBERT A., Professor of Zoölogy, Oberlin College.

CALKINS, GARY N., Professor of Protozoölogy, Columbia University.

CAROTHERS, E. ELEANOR, University of Pennsylvania.

CHAMBERS, ROBERT, Professor of Histology and Embryology, Cornell University Medical College.

CHARLTON, HARRY H., Assistant Professor of Anatomy, University of Missouri.

CLAPP, CORNELIA M., Professor Emeritus of Zoölogy, Mount Holyoke, College.

CLARK, ELIOT R., Professor of Anatomy, University of Missouri.

CLARK, ELEANOR L., Columbia, Mo.

COLE, WILLIAM H., Professor of Biology, Lake Forest College.

CONKLIN, EDWIN G., Professor of Biology, Princeton University. ·

COPELAND, MANTON, Professor of Biology, Bowdoin College.

COWDRY, EDMUND V., Associate Member, Rockefeller Institute.

COWDRY, NATHANIEL H., 1142 Madison Ave., New York City.

DANCHAKOFF, VERA, Assistant Professor of Anatomy, College of Physicians and Surgeons.

DAWSON, JAMES A., Professor of Biology, Dalhousie University.

DOLLEY, DAVID H., Professor, University of Missouri.

DOLLEY, WILLIAM L., JR., Professor of Biology, Randolph-Macon College.

DONALDSON, HENRY H., Professor of Neurology, Wistar Institute.

DREW, GILMAN A., Assistant Director, Marine Biological Laboratory, Woods Hole, Mass.

GLASER, OTTO, Professor of Biology, Amherst College.

GOLDFARB, A. J., Assistant Professor, College of the City of New York.

GOLDSMITH, WILLIAM M., Professor of Biology, Southwestern College.

GOODRICH, H. B., Associate Professor of Zoölogy, Wesleyan University.

GRAVE, BENJAMIN H., Professor of Zoölogy, Wabash College.

HEILBRUNN, LEWIS V., Assistant Professor of Zoölogy, University of Michigan.

HESS, WALTER N., Professor of Biology, DePauw University.

HIBBARD, HOPE, Associate Professor of Zoölogy, Elmira College.

HOWE, HARRISON E., 2702 36th St., N. W., Washington, D. C.

HUETTNER, ALFRED F., Instructor, Columbia University.
HUMPHREY, RUFUS R., Instructor, Cornell University.
JACOBS, MERKEL H., Assistant Professor of Zoölogy, University of Pennsylvania.
JENNINGS, HERBERT S., Professor of Zoölogy, Johns Hopkins University.
JUST, ERNEST E., Professor of Zoölogy, Howard University.
KNOWER, HENRY McE., Professor of Anatomy, University of Cincinnati.
LANCEFIELD, DONALD E., Assistant Professor of Zoölogy, Columbia University.
LEFEVRE, GEORGE, Professor of Zoölogy, University of Missouri.
LEWIS, WARREN H., Collaborator, Carnegie Institution of Washington.
LEWIS, MARGARET R., Collaborator, Carnegie Institution of Washington.
LILLIE, FRANK R., Chairman, Department of Zoölogy, University of Chicago.
LYNCH, RUTH S., Assistant, Johns Hopkins University.
McCLUNG, CLARENCE E., Director of Zoölogical Laboratory, University of Pennsylvania.
McEWEN, ROBERT S., Assistant Professor of Zoölogy, Oberlin College.
MADDOCK, STEPHEN J., Teaching Fellow in Histology, Harvard University Medical School.
MALONE, EDWARD F., Professor of Histology, University of Cincinnati.
MARTIN, EARL A., Assistant Professor, College of the City of New York.
MAST, SAMUEL O., Professor of Zoology, Johns Hopkins University.
METZ, CHARLES W., Member of Staff, Carnegie Institution, Cold Spring Harbor.
MOORE, EMILY L., Research Fellow, Yale University.
MORGAN, THOMAS H., Professor of Experimental Zoölogy, Columbia University.
MORGAN, ANN H., Professor of Zoölogy, Mount Holyoke College.
MORGAN, LILIAN V., 409 West 117th St., New York City.
NOYES, BESSIE, North Carolina College for Women.
PARKER, GEORGE H., Professor of Zoölogy, Harvard University.
PARMENTER, CHARLES L., Instructor, University of Pennsylvania.
PATTEN, WILLIAM, Professor of Biology, Dartmouth College.
PLOUGH, HAROLD H., Associate Professor of Biology, Amherst College.
POTTS, FRANK A., Lecturer in Zoölogy, University of Cambridge.
SCHRADER, FRANZ, Bryn Mawr College.
SHUMWAY, WALDO, Assistant Professor of Biology, Dartmouth College.
SIVICKIS, P. B., University of Chicago.
SMITH, ELIZABETH A., Assistant Professor of Zoölogy, University of Wisconsin.
SPAULDING, EDWARD G., Professor of Philosophy, Princeton University.
SPEIDEL, CARL C., Assistant Professor of Anatomy, University of Virginia.
STARK, MARY B., Professor of Embryology and Histology, New York Hospital Medical College.
STRONG, OLIVER S., Associate Professor of Neurology, Columbia University.
STURTEVANT, ALFRED H., Research Assistant, Carnegie Institution of Washington.
SWETT, FRANCIS H., Instructor in Anatomy, Johns Hopkins University Medical School.
SWINGLE, WILBUR W., Assistant Professor of Biology, Yale University.
SYKES, GEORGE F., Teaching Fellow, Harvard University Medical School.
TENNENT, DAVID H., Professor of Biology, Bryn Mawr College.
TRACY, HENRY C., Professor of Anatomy, University of Kansas.
WEINSTEIN, ALEXANDER, Research Fellow, Columbia University.
WIEMAN, HARRY L., Professor of Zoölogy, University of Cincinnati.
WOODRUFF, LORANDE L., Professor of Biology, Yale University.

WOODWARD, ALVALYN E., Amherst College.
YOUNG, BENJAMIN P., Assistant Professor of Zoölogy, Cornell University.
YOUNG, DONNELL B., Assistant Professor of Biology, Carleton College.

BEGINNING INVESTIGATORS—Zoölogy.

BEAN, RAYMOND J., Instructor in Biology, Western Reserve University.
BENNITT, RUDOLPH, Teaching Fellow, Harvard University.
BRAILEY, MIRIAM E., Assistant in Embryology, Mount Holyoke College.
BROWN, ALICE L., Assistant in Anatomy, Cornell University Medical College.
CHACE, EUNICE E., Instructor, Smith College.
CHASE, RUTH W., Assistant in Zoölogy, University of Wisconsin.
COLE, ELBERT C., Trinity College.
EMERSON, STERLING H., Carnegie Institution, Cold Spring Harbor.
FERRY, RUTH M., Assistant, Carnegie Institution, Cold Spring Harbor.
FRY, HENRY J., Columbia University.
GILSON, ARTHUR S., JR., Research Student, Harvard University.
HAYDEN, MARGARET A., Instructor in Zoölogy, Wellesley College.
HINRICHS, MARIE A., University of Chicago.
HOADLEY, LEIGH, Assistant, University of Chicago.
JOHNSON, H. HERBERT, Laboratory Assistant in Zoölogy, Columbia University.
JOHNSON, GEORGE E., Graduate Student, Harvard University.
LACKEY, JAMES B., Instructor in Zoölogy, Mississippi College.
LOWMAN, EDITH, Assistant Instructor, Yale University.
LOWTHER, FLORENCE DEL., Instructor, Barnard College.
MACBRIDE, LAVINIA G., Graduate Student, University of Michigan.
MACDOUGALL, MARY S., Head of Biology Dept., Agnes Scott College.
MOSES, MILDRED S., Assistant, Carnegie Institution, Cold Spring Harbor.
OVERSTREET, MRS. HARRY A., 802 West 181st St., New York City.
SMITH, CHRISTIANNA, Graduate Fellow, Cornell University.
STURTEVANT, PHOEBE R., Carnegie Institution of Washington.
THARALDSEN, CONRAD E., Assistant Professor of Zoölogy, Northwestern University.
THATCHER, LLOYD E., Instructor in Zoölogy, University of Michigan.
UHLEMEYER, BERTHA, Instructor in Zoölogy, Washington University.
VARIAN, BASIL B., Columbia University.
VICARI, EMILIA, Columbia University.
VISSCHER, J. PAUL, Fellow, Washington University.
WALLACE, EDITH M., Carnegie Institution.
WARREN, HERBERT S., Assistant in Zoölogy, Columbia University.
WILLIAMS, STEPHEN C., Wesleyan University.

INDEPENDENT INVESTIGATORS—Physiology.

BISHOP, GEORGE H., Associate, Washington University Medical College.
BRADLEY, HAROLD C., Professor of Physiological Chemistry, University of Wisconsin.
CLOWES, GEORGE H. A., Research Director, Eli Lilly & Co., Indianapolis, Ind.
COLLETT, MARY E., Instructor in Physiology, University of Buffalo.
COHN, EDWIN J., Assistant Professor of Physiological Chemistry, Harvard University Medical School.
DENIS, WILLY, Associate Professor of Physiological Chemistry, Tulane University.

EDWARDS, DAYTON J., Associate Professor of Physiology, Cornell University Medical College.

GARREY, WALTER E., Professor of Physiology, Tulane University.

GEISER, SAMUEL W., Assistant Professor of Zoölogy, Washington University.

GREISHEIMER, ESTHER M., Instructor in Physiology, University of Minnesota.

HARVEY, E. NEWTON, Professor of Physiology, Princeton University.

HECHT, SELIG, National Research Council Fellow, Harvard University.

HITCHCOCK, DAVID I., Assistant, Rockefeller Institute for Medical Research.

HOPKINS, HOYT S., Assistant Professor of Physiology, Baylor University Medical College.

IRWIN, MARIAN, Research Worker, Radcliffe College.

KNOWLTON, FRANK P., Professor of Physiology, Syracuse University.

LILLIE, RALPH S., Biologist, Nela Research Laboratories, Cleveland, Ohio.

LOEB, JACQUES, Head of Division of Experimental Biology, Rockefeller Institute for Medical Research.

LOEB, LEO, Professor of Comparative Pathology, Washington University.

MOORE, ARTHUR R., Professor of Physiology, Rutgers College.

MORGULIS, SERGIUS, Professor of Biochemistry, University of Nebraska, College of Medicine.

POND, SAMUEL E., Biologist, Nela Research Laboratories, Cleveland, Ohio.

RAPPORT, ANNE Y., Associate in Physiology, Bryn Mawr College.

REDFIELD, ALFRED C., Assistant Professor of Physiology, Harvard University Medical School.

SMITH, HOMER W., Eli Lilly & Co., Indianapolis, Ind.

SPAETH, REYNOLD A., Associate in Physiology, School of Public Health, Johns Hopkins University.

SPARROW, CARROLL M., Professor of Physics, University of Virginia.

WARREN, HOWARD C., Professor of Psychology, Princeton University.

BEGINNING INVESTIGATORS—Physiology.

BIERMAN, JESSIE M., Medical Student, University of Chicago.

BRIGHT, ELIZABETH M., Research Assistant in Physiology, Harvard University Medical School.

BURLINGHAM, ROBERT, 156 East 66th St., New York City.

CATTELL, WARE, Garrison, N. Y.

HENDRY, JESSIE L., Technician, Harvard University Medical School.

KLOPP, JOHN W., University of Pennsylvania, School of Medicine.

PAGE, IRVINE H., Chemist, Eli Lilly & Co., Indianapolis, Ind.

SAMPSON, MYRA M., Assistant Professor of Zoölogy, Smith College.

SHEPARD, CHARLES E., Teaching Fellow, University of Minnesota.

STUDEBAKER, MABEL T., Eli Lilly & Co., Indianapolis, Ind.

WALDEN, EDA B., Chemist, Eli Lilly & Co., Indianpaolis, Ind.

INDEPENDENT INVESTIGATORS—Botany.

BROOKS, SUMNER C., Hygienic Laboratory, United States Public Health Service.

BROOKS, MRS. S. C., Hygienic Laboratory, United States Public Health Service.

CLELAND, RALPH E., Assistant Professor of Biology, Goucher College.

EAST, EDWARD M., Professor of Experimental Plant Morphology, Harvard University.

KYLIN, HAROLD, Lund, Sweden.
LEWIS, IVEY F., Professor of Biology, University of Virginia.
LYMAN, GEORGE R., U. S. Department of Agriculture, Washington, D. C.
MOORE, GEORGE T., Director Missouri Botanical Gargen, St. Louis, Mo.
OSTERHOUT, WINTHROP J. V., Professor of Botany, Harvard University.
PHILLIPS, EVERETT F., U. S. Department of Agriculture, Washington, D. C.
RAY, GEORGE B., Instructor in Applied Physiology, Harvard University Medical School.
SCHRAMM, J. R., Professor of Botany, Cornell University.
SNOW, LAETITIA M., Associate Professor of Botany, Wellesley College.
TAYLOR, WILLIAM R., Assistant Professor of Botany, University of Pennsylvania.
WESTON, WILLIAM H., JR., Assistant Professor of Botany, Harvard University.

BEGINNING INVESTIGATORS—Botany.

COOK, SHERBURNE F., Teaching Fellow, Harvard University.
HARRIS, EARL S., Harvard University.
HARVEY, PAUL A., Teaching Fellow, Harvard University.
KEEFE, ANSELM M., Instructor, St. Norbert's College.
LYON, CHARLES J., Instructor in Biology, Dartmouth College
MACINNES, JEAN, Massachusetts Institute of Technology.
MACKAYE, ROBERT K., Student, Harvard University.

STUDENTS.

1922.

BOTANY.

BRISTOL, MARY L., Student, Connecticut College.
EASTON, CHARLOTTE, Head of Biology Dept., Skidmore College.
HESS, FLORENCE G., Student, Cornell University
MCNAIR, MRS. GEORGE T., Chickasha, Okla.
NUGENT, GERTRUDE V., Teacher, East Boston, Mass.
PAGE, HELEN D., Student, University of Chicago.
SMITH, FANNY FERN, Student, Washington University
WOODHEAD, ARTHUR E., Professor of Biology, Western Maryland College.

EMBRYOLOGY.

ANDERSON, PEARL, Assistant in Zoölogy, Vassar College.
BRADLEY, LILLIAN H., Columbia University.
BROWN, LOUISE K., Student, Agnes Scott College.
BUTLER, ELMER G., Instructor in Zoölogy, University of Vermont.
CHAMBERLAIN, WILLIAM H., Columbia University.
CHANG, C., Student, Harvard Medical School.
HALLAUER, EMILY E., Swarthmore College.
HALTER, CLARENCE R., Instructor in Zoölogy, New York University.
HAWKINS, MARY O'NEIL, 1331 Columbine St., Denver, Colorado.
HULPIEU, HAROLD R., Student, Southwestern College.
LEVY, JOSEPH, Student, Johns Hopkins University.
LILLIE, MARGARET H., Student, Mount Holyoke College.

LUCAS, ALFRED M., Student, Wabash College.
McCAA, FANNY, Instructor, Agnes Scott College.
METCALF, RACHEL V., Instructor in Zoölogy, Mount Holyoke College.
MILLER, HARRY M., JR., Fellow in Zoölogy, University of Illinois.
MILLER, FRANKLIN R., Student, Illinois Wesleyan University
PIERSON, CHARLES J., Professor of Zoölogy, University of Maryland.
ROBINSON, OWEN L., Student, De Pauw University.
SHAVER, JESSE M., Assistant Professor of Biology, George Peabody College.
STEEN, EDWIN B., Assistant in Zoölogy, Wabash College.
STEVENS, EDITH, West Virginia University.
STEWART, COLIN C., JR., Student, Dartmouth College.
STEWART, DOROTHY R., Assistant, Washington University.
TOWLER, VIOLA, Shorter College.
WASSUM, EVA E., Student, Agnes Scott College.
WICKS, NINA A., Student, Knox College.
WILCOX, GLADYS, University of Delaware.

PHYSIOLOGY.

BROWN, MADELAINE R., Social Worker, State Hospital for Mental Diseases, Howard, R. I.
BROWN, MARY J., Associate Professor, Transylvania College.
COVENTRY, FRANCES A., Assistant, Biology Dept., Goucher College.
DICKINSON, PORTER S., Student, Harvard Medical School.
DIMICK, G. PRISCILLA, Smith College.
DRAKE, DOROTHY, Assistant in Physiology, Mount Holyoke College.
FLEXNER, LOUIS B., Student, University of Chicago.
GILMAN, CHARLOTTE W., Instructor in Zoölogy, Vassar College.
HARTMAN, ARTHUR M., 1414 Girard St., N. W., Washington, D. C.
ISZARD, MIRIAM S., Instructor in Bacteriology, University of Pennsylvania.
LANDIS, EUGENE M., Student, University of Pennsylvania.
LEWTON, LUCY O., Barnard College.
LINDSAY, BLANCHE, Assistant in Zoölögy and Physiology, Wellesley College.
MORRISON, THOMAS F., Student, Princeton University.
PATCH, ESTHER M., Teacher, Stoneham, Mass.
REYNOLDS, PHILIP A., Boston University School of Medicine.
SLOAN, LOUISE L., Student, Bryn Mawr College.
WHITE, E. GRACE, Professor of Biology, Shorter College.
WOOLLARD, HERBERT H., Lecturer in Anatomy, University College, London.

PROTOZOÖLOGY.

BAKER, W. B., Assistant Professor of Biology, Emory University.
BOX, CORA M., Assistant Professor, University of Cincinnati.
BOYD, GEORGE H., Instructor in Biology, Emory University.
COUTANT, MARY W., Instructor in Botany, Barnard College.
EMMART, EMILY W., 817 N. Fremont Ave., Baltimore, Md.
GRAVES, ISABELLE A., Assistant Bacteriologist, City Laboratory, New Haven, Conn.
NOLAND, LOWELL E., Instructor, Zoology Dept., University of Wisconsin.
SCHRADER, SALLY H., Bryn Mawr, Pa.

TAO, WEI SUN, Student, Columbia University.
TILDEN, EVELYN B., Rockefeller Institute.
UNGER, W. BYERS, Instructor in Biology, Lafayette College.
WALTERS, MARY J., Instructor, Goucher College.

ZOÖLOGY.

ANDERSON, ETHEL L., University of Kentucky.
ANDERSON, HOPE E., Student, Mount Holyoke, College.
BAMBER, MAURINE, Student, Knox College.
BATON, GERTRUDE M., Assistant Instructor in Biology, Carnegie Institute of Technology.
BROWN, BERNICE D., Student, Oberlin College.
BUEHLER, EUGENE O., Student, Wabash College.
BURWELL, MARGARET S., Student, Sweet Briar College.
CAMPBELL, EVA G., Instructor, North Carolina College for Women.
COOPER, DRURY W., JR., Student, Rutgers College.
COPENHAVER, WILFRED M., Assistant in Biology, Yale University.
DENBY, EDWIN O., Student, Harvard College.
DIXON, PERRINE C., Student, Sophie Newcomb College.
DOOLEY, PARKER, Student, Illinois Wesleyan University.
FAW, HELEN A., Student, Agnes Scott College.
FEDERIGHI, HENRY, Student, Rutgers College.
FULLER, ANDREW B., Student, University of Pennsylvania.
GORHAM, GRACE V., Undergraduate student, Mount Holyoke College.
GRANT, JEAN F., Student, Sweet Briar College.
GRAY, IRVING E., Instructor, DePauw University.
GRAY, NINA E., Student, DePauw University.
HAGEDON, EDITH L., Student, DePauw University.
HALL, FRANK G., Assistant in Zoölogy, University of Wisconsin.
HOPKINS, AUBREY E., College of William and Mary.
HOUGHTON, DOROTHY, Student, Barnard College.
HUBER, ERNST, Associate in Anatomy, Johns Hopkins Medical School.
HUNSICKER, MARY G., Student, University of Pennsylvania.
JONES, MYRNA F., Student, Doane College.
KENNAN, ADA B., Assistant in Zoölogy, University of Michigan.
KUBICEK, HELEN M., Student, Doane College.
LEWIS, MARION F., Student, Mount Holyoke College.
LUCE, ROBERT H., Carleton College.
MAGUIRE, GERTRUDE A., Student, Hunter College.
MASON, KARL E., Assistant in Zoölogy, Yale University.
McNAIR, GEORGE T., Professor of Zoölogy, Oklahoma College for Women.
MIEHLING, RUTH B., Student, Hunter College.
MILLER, GLENN O., Student, Southwestern College.
MORGAN, RUBY M., Student, Oberlin College.
NICKELL, FAITH E., Washington University.
NORTON, HERMON, Student, Wesleyan University.
NYI, TSUNG-TSONG, Student, Smith College.
PAYNE, MARY G., Student, Butler College.
PERRY, LYDIA S., Student, Oberlin College.
PRITCHARD, MAYBELLE, Student, Radcliffe College.

REED, LUCILE L., Student, Sophie Newcomb College.
RILEY, PHILIP L., Student, Massachusetts Institute of Technology.
ROSE, BERTHA E., Assistant in Zoölogy, University of Wisconsin.
RUSTIA, CONSTANCIO P., Graduate Student, University of Chicago.
SAMPSON, HOWARD J., Massachusetts Agricultural College.
SCOTT, GORDON H., Assistant, Johns Hopkins University.
SOISSON, MARY C., Goucher College.
STEVENS, DOROTHY H., Student, Connecticut College.
STIFFLER, ETHEL G., Student, Goucher College.
STRAUSS, MAURICE B., Student, Amherst College.
SUMWALT, MARGARET, Student, Goucher College.
TAFT, CHARLES H., JR., Instructor in Biology, Tufts College.
TAYLOR. ELIZABETH R., University of Delaware.
THEOCHARIDES, ELECTRA, Assistant in Biology, Constantinople College.
TINGLEY, MARY A., Instructor, Shorter College.
VAN HORN, AMEY D., Instructor in Biology, Alfred University.

3. TABULAR VIEW OF ATTENDANCE.

	1918	1919	1920	1921	1922
INVESTIGATORS—Total	93	134	136	172	182
Independent:					
Zoölogy	51	68	69	79	87
Physiology	16	24	22	26	28
Botany	5	7	7	13	15
Under Instruction:					
Zoölogy	16	21	29	34	34
Physiology	3	10	7	11	11
Botany	2	4	2	9	7
STUDENTS—Total	69	128	120	120	126
Zoölogy	41	55	56	53	59
Protozoölogy	—	15	15	11	12
Embryology	12	33	26	28	28
Physiology	10	17	15	18	19
Botany	6	8	8	10	8
TOTAL ATTENDANCE	162	262	256	292	308
INSTITUTIONS REPRESENTED—					
Total	72	88	86	95	104
By investigators	49	61	55	67	71
By students	38	62	57	53	68
SCHOOLS AND ACADEMIES REPRESENTED.					
By investigators	—	—	1	—	—
By students	—	4	7	—	—

4. SUBSCRIBING AND COÖPERATING INSTITUTIONS IN 1922.

AMHERST COLLEGE.
BARNARD COLLEGE.
BOWDOIN COLLEGE.
BRYN MAWR COLLEGE.
BUTLER COLLEGE.
CARNEGIE INSTITUTION OF WASHINGTON.
CARNEGIE INSTITUTION, COLD SPRING HARBOR.
CARNEGIE INSTITUTE OF TECHNOLOGY.
COLLEGE OF PHYSICIANS AND SURGEONS.
COLUMBIA UNIVERSITY.
CONNECTICUT COLLEGE.
CORNELL UNIVERSITY.
CORNELL UNIVERSITY MEDICAL COLLEGE.
DARTMOUTH COLLEGE.
DEPAUW UNIVERSITY.
DOANE COLLEGE.
ELI LILLY & CO.
GOUCHER COLLEGE.
HARVARD UNIVERSITY.
HARVARD UNIVERSITY MEDICAL SCHOOL.
HUNTER COLLEGE.
ILLINOIS WESLEYAN UNIVERSITY.
JOHNS HOPKINS UNIVERSITY.
JOHNS HOPKINS UNIVERSITY MEDICAL SCHOOL.
KNOX COLLEGE.
LAKE FOREST COLLEGE.
MASSACHUSETTS INSTITUTE OF TECHNOLOGY.
MOUNT HOLYOKE COLLEGE.

NELA RESEARCH LABORATORIES.
NORTH CAROLINA COLLEGE FOR WOMEN.
OBERLIN COLLEGE.
PRINCETON UNIVERSITY.
RADCLIFFE COLLEGE.
ROCKEFELLER FOUNDATION.
ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH.
RUTGERS COLLEGE.
SOPHIE NEWCOMB COLLEGE.
SOUTHWESTERN COLLEGE.
SWEET BRIAR COLLEGE.
TUFTS COLLEGE.
U. S. VETERANS BUREAU.
UNIVERSITY OF CHICAGO.
UNIVERSITY OF CINCINNATI.
UNIVERSITY OF DELAWARE.
UNIVERSITY OF ILLINOIS.
UNIVERSITY OF KANSAS.
UNIVERSITY OF MARYLAND.
UNIVERSITY OF MICHIGAN.
UNIVERSITY OF MINNESOTA.
UNIVERSITY OF PENNSYLVANIA.
UNIVERSITY OF THE PHILIPPINES.
UNIVERSITY OF VERMONT.
UNIVERSITY OF WISCONSIN.
VASSAR COLLEGE.
WABASH COLLEGE.
WASHINGTON UNIVERSITY.
WESLEYAN UNIVERSITY.
WELLESLEY COLLEGE.
WESTERN RESERVE UNIVERSITY.
WISTAR INSTITUTE OF ANATOMY AND BIOLOGY.
YALE UNIVERSITY.

SCHOLARSHIP TABLES.

THE LUCRETIA CROCKER SCHOLARSHIPS FOR TEACHERS IN BOSTON, SINCE 1888.

SCHOLARSHIP OF $100.00, SUPPORTED BY A FRIEND OF THE LABORATORY, SINCE 1898.

THE NEW LONDON BRANCH OF THE AMERICAN ASSOCIATION OF UNIVERSITY WOMEN, SINCE 1920.

5. EVENING LECTURES, 1922.

Friday, June 30,
DR. SELIG HECHT . . . . "The Visibility of the Spectrum."

Friday, July 7,
DR. H. S. JENNINGS . . . . "A Critical Study of the So-called Linear Theory of Crossing-Over in Inheritance."

Tuesday, July 11,
DR. D. H. DOLLEY . . . . "Specific Functions and Specific Irritability."

Friday, July 14,
DR. GEORGE SARTON . . . . "The History of Science."

Tuesday, July 18,
DR. E. E. JUST . . . . "Certain Effects of Hypertonic Sea-water in Activating *Arbacia* Eggs."

Friday, July 21,
MR. WM. LYMAN UNDERWOOD . . "Wild Brother."

Monday, July 24,
DR. J. A. DETLEFSEN . . . . "Experiments Regarding the Inheritance of the Effects of Rotation in Rats."

Tuesday, July 25,
DR. C. B. BRIDGES . . . . "Chromosomes and Inheritance in *Drosophila melanogaster*."

Friday, July 28,
DR. G. H. PARKER . . . . "Physiology of Actinian Muscle."

Monday, July 31,
DR. W. H. WESTON . . . . "Following a Fungus through the Philippines."

Tuesday, August 1,
DR. W. H. LEWIS . . . . "Tissue Culture."

Friday, August 4,
DR. J. R. SCHRAMM . . . . "Some Thoughts on Abstracting and Indexing Biological Literature."

Tuesday, August 8,
DR. W. D. BANCROFT . . . . "Structural Colors in Feathers."

6. MEMBERS OF THE CORPORATION.

1. LIFE MEMBERS.

ALLIS, MR. E. P., JR., Palais Carnoles, Menton, France.

ANDREWS, MRS. GWENDOLEN FOULKE, Baltimore, Md.

BILLINGS, MR. R. C., 66 Franklin St., Boston, Mass.

CAREY, MR. ARTHUR ASTOR, Fayerweather St., Boston, Mass.

CLARKE, PROF. S. F., Williamstown, Mass.

CONKLIN, PROF. EDWIN G., Princeton University, Princeton, N. J.

CRANE, MR. C. R., Woods Hole, Mass.

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FOLSOM, MISS AMY, 88 Marlboro St., Boston, Mass.

FOOT, MISS KATHERINE, Care of Morgan Harjes Cie, Paris, France.

GARDINER, MRS. E. G., Woods Hole, Mass.

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KING, MR. CHAS. A.

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MINNS, MR. THOMAS, 14 Louisburg Square, Boston, Mass.

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MORGAN, MRS. T. H., New York City, N. Y.

NOYES, MISS EVA J.

NUNN, MR. LUCIAN L., Telluride, Colo.

OSBORN, PROF. HENRY F., American Museum of Natural History, New York City, N. Y.

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PHILLIPS, MRS. JOHN C., Windy Knob, Wenham, Mass.

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PULSIFER, MR. W. H., Newton Center, Mass.

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SHEDD, MR. E. A.

THORNDIKE, DR. EDWARD L., Teachers College, Columbia University, New York City, N. Y.

TRELEASE, PROF. WILLIAM, University of Illinois, Urbana, Ill.

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WHITNEY, MR. HENRY M., Brookline, Mass.

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WILSON, DR. E. B., Columbia University, New York City, N. Y.

WILSON, PROF. W. P., Commercial Museum, Philadelphia, Pa.

2. REGULAR MEMBERS, AUGUST, 1922.

ADAMS, MISS A. E., Mount Holyoke College, South Hadley, Mass.

ADDISON, DR. W. H. F., University of Pennsylvania Medical School, Philadelphia, Pa.

ADOLPH, DR. EDWARD F., University of Pittsburgh, Pittsburgh, Pa.

AGERSBORG, DR. H. P. K., University of Nebraska, Lincoln, Neb.

ALLEE, DR. W. C., University of Chicago, Chicago, Ill.

ALLEN, PROF. EZRA, Ursinus College, Collegeville, Pa.

ALLYN, MISS HARRIET M., Hackett Medical College, Canton, China.

ALTENBURG, DR. EDGAR, Rice Institute, Houston, Texas.

ANDERSON, DR. E. G., College of the City of New York, New York City.

ATTERBURY, MRS. RUTH R., College of Physicians and Surgeons, New York City, N. Y.

BAITSELL, DR. GEORGE A., Yale University, New Haven, Conn.

BAKER, MRS. L. D., 123 Chiswick Road, Boston, Mass.

BAKER, DR. E. H., 5729 Kimbark Ave., Chicago, Ill.

BALDWIN, DR. F. M., Iowa State College, Ames, Iowa.

BANCROFT, PROF. F. W., Aloha Farm, Concord, Calif.

BASCOM, DR. K. F., Allegheny College, Meadville, Pa.

BECKWITH, DR. CORA J., Vassar College, Poughkeepsie, N. Y.

BEHRE, DR. ELINOR H., Louisiana State University, Baton Rouge, La.

BIGELOW, PROF. M. A., Teachers College, Columbia University, New York City, N. Y.

BIGELOW, PROF. R. P., Massachusetts Institute of Technology, Cambridge, Mass.

BINFORD, PROF. RAYMOND, Guilford College, Guliford College, N. C.

BORING, DR. ALICE M., Wellesley College, Wellesley, Mass.

BOX, MISS CORA MAY, University of Cincinnati, Cincinnati, Ohio.

BOWEN, DR. ROBERT H., Columbia University, New York City, N. Y.

BRADLEY, PROF. HAROLD C., University of Wisconsin, Madison, Wis.

BRAILEY, MISS MIRIAM E., Mount Holyoke College, South Hadley, Mass.

BRIDGES, DR. CALVIN B., Columbia University, New York City, N. Y.

BROOKS, DR. S. C., U. S. Public Health Service, Washington, D. C.

BRUMFIEL, DR. DANIEL M., University of Iowa, Iowa City, Iowa.

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BUDINGTON, PROF. R. A., Oberlin College, Oberlin, Ohio.

BUMPUS, DR. H. C., Brown University, Providence, R. I.

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CALKINS, PROF. GARY N., Columbia University, New York City, N. Y.

CALVERT, PROF. PHILIP P., University of Pennsylvania, Philadelphia, Pa.

CARLSON, PROF. A. J., University of Chicago, Chicago, Ill.

CAROTHERS, DR. ELEANOR E., University of Pennsylvania, Philadelphia, Pa.

CARPENTER, PROF. FREDERIC W., Trinity College, Hartford, Conn.

CARROLL, PROF. MITCHEL, Franklin and Marshall College, Lancaster, Pa.

CARVER, PROF. GAIL L., West Lake, Ga.

CASEY, COLONEL THOMAS L., Washington, D. C.

CASTEEL, DR. D. B., University of Texas, Austin, Texas.

CATTELL, PROF. J. McKEEN, Garrison-on-Hudson, N. Y.

CATTELL, MR. McKEEN, Harvard Medical School, Boston, Mass.

CHAMBERS, DR. ROBERT, Cornell University Medical College, New York City, N. Y.

CHARLTON, DR. HARRY H., University of Missouri, Columbia, Mo.

CHIDESTER, PROF. F. E., West Virginia University, Morgantown, W. Va.

CHILD, PROF. C. M., University of Chicago, Chicago, Ill.

CLAPP, PROF. CORNELIA M., Mount Holyoke College, South Hadley, Mass.

CLARK, PROF. E. R., University of Georgia, Augusta, Ga.

CLOWES, DR. G. H. A., Eli Lilly & Co., Indianapolis, Ind.

COE, PROF. W. R., Yale University, New Haven, Conn.

COHN, DR. EDWIN J., 25 Follen St., Cambridge, Mass.

COLE, DR. LEON J., College of Agriculture, Madison, Wis.

COLLETT, DR. MARY E., Ashland, Mass.

COLTON, PROF. H. S., Ardmore, Pa.

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COPELAND, PROF. MANTON, Bowdoin College, Brunswick, Maine.

COUTANT, MRS. MARY W., Barnard College, Columbia University, New York City, N. Y.

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CRAMPTON, PROF. H. E., Barnard College, Columbia University, New York City, N. Y.

CRANE, MRS. C. R., Woods Hole, Mass.

CURTIS, PROF. W. C., University of Missouri, Columbia, Mo.

CURTIS, DR. MAYNIE R., Crocker Laboratory, Columbia University, New York City, N. Y.

DANCHAKOFF, DR. VERA, College of Physicians and Surgeons, New York City, N. Y.

DAVIS, DR. DONALD W., College of William and Mary, Williamsburg, Va.

DAVIS, PROF. BRADLEY M., University of Michigan, Ann Arbor, Mich.

DAVIS, DR. ALICE R., Harvard Medical School, Boston, Mass.

DAWSON, DR. J. A., Dalhousie University, Halifax, Nova Scotia.

DEDERER, DR. PAULINE H., Connecticut College, New London, Conn. ·

DEXTER, DR. J. S., Northwestern College, Naperville, Ill.

DODDS, PROF. G. S., Medical School, University of West Virginia, Morgantown, W. Va.

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DUNN, DR. ELIZABETH H., Woods Hole, Mass.

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ELLIS, DR. F. W., Monson, Mass.

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FIELD, MISS HAZEL E., Sophie Newcomb College, New Orleans, La.

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GAGE, PROF. S. H., Cornell University, Ithaca, N. Y.

GARREY, PROF. W. E., Tulane University, Richardson Memorial, New Orleans, La.

GATES, PROF. R. RUGGLES, University of London, London, England.

GLASER, PROF. O. C., Amherst College, Amherst, Mass.

GLASER, PROF. R. W., Rockefeller Institute for Medical Research, Princeton, N. J.

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GRAVE, PROF. CASWELL, Washington University, St. Louis, Mo.

GRAVE, PROF. B. H., Wabash College, Crawfordsville, Ind.

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HARGITT, PROF. GEORGE T., Syracuse University, Syracuse, N. Y.

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HARRISON, PROF. Ross G., Yale University, New Haven, Conn.

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HEATH, PROF. HAROLD, Palo Alto, Calif.

HEGNER, PROF. R. W., Johns Hopkins University, Baltimore, Md.

HEILBRUNN, DR. L. V., University of Michigan, Ann Arbor, Mich.

HESS, PROF. WALTER N., DePauw University, Greencastle, Ind.

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HOPKINS, DR. HOYT S., Baylor University, Dallas, Texas.

HOSKINS, MRS. ELMER R., University of Arkansas Medical School, Little Rock, Ark.

HOWE, DR. H. E., National Research Council, Washington, D. C.

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•JACKSON, PROF. C. M., University of Minnesota, Minneapolis, Minn.

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JUST, PROF. E. E., Howard University, Washington, D. C.

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KNOWLTON, PROF. F. P., Syracuse University, Syracuse, N. Y.

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KRIBS, DR. HERBERT, University of Pennsylvania, Philadelphia, Pa.

KUYK, DR. MARGARET P., Richmond, Va.

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LANGE, DR. MATHILDE M., Wheaton College, Norton, Mass.

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LINTON, PROF. EDWIN, 1104 Milledge Road, Augusta, Ga.

LOEB, PROF. JACQUES, Rockefeller Institute for Medical Research, New York City, N. Y.

LOEB, PROF. LEO, Washington University Medical School, St. Louis, Mo.

LOEB, MRS. LEO, St. Louis, Mo.

LOWTHER, MRS. FLORENCE DEL., Barnard College, Columbia University, New York City, N. Y.

LUND, DR. E. J., University of Minnesota, Minneapolis, Minn.

LUSCOMBE, MR. W. O., Woods Hole, Mass.

LYMAN, PROF. GEORGE R., Plant Disease Survey, Department of Agriculture, Washington, D. C.

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McGEE, DR. A. N., Woods Hole, Mass.

McGILL, DR. CAROLINE, Murray Hospital, Butte, Montana.

McGREGOR, DR. J. H., Columbia University, New York City, N. Y.

McINDOO, DR. N. E., Bureau of Entomology, Washington, D. C.

McMURRICH, PROF. J. P., University of Toronto, Toronto, Canada.

MACKLIN, DR. CHARLES C., School of Medicine, Western University, London, Canada.

MALONE, PROF. E. F., University of Cincinnati, Cincinnati, Ohio.

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MAST, PROF. S. O., Johns Hopkins University, Baltimore, Md.

MATHEWS, PROF. A. P., University of Cincinnati, Cincinnati, Ohio.

MATSUI, PROF. K., Imperial College of Agriculture and Dendrology, Morioka, Japan.

MAVOR, PROF. JAMES W., Union College, Schenectady, N. Y.

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SEX–DIFFERENTIATION IN THE VIVIPAROUS TELEOST *XIPHOPHORUS HELLERI* HECKEL.

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CONTENTS.

I. INTRODUCTION.

In the fall of 1920, Dr. A. W. Bellamy, who is carrying on breeding experiments with several viviparous teleosts in this laboratory, obtained two *Xiphophorus helleri* from F. E. Boehn, a member of the Chicago Aquarium Club, who claimed that these fish had been subject to sex-inversion. According to this report he had three unusually large specimens—two females and one male—which he kept in a breeding tank and from which he raised more than five hundred young. When the females were about

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three years old they ceased producing young and, during the course of several weeks, took on the secondary sex characters of males.

When, a few days later, these specimens came into the writer's possession, both were decidedly female as to shape and size of body and decidedly male as to development of anal and caudal fins, which were far advanced toward the male condition yet much belated. Cytological preparations of the entire reproductive system of both fishes disclosed that ripe sperm was in all parts of the duct and gonad, but that the latter was juvenile in comparison to the size and age of the fish.

Suggestive as the case was, the data obtained were far too meager to decide either for or against the reputed transformation. That peculiarities do occur in the sex conditions of these teleosts has been noted in this laboratory as well as by many fish fanciers. The extent and meaning of these peculiarities became the aim of this work. It was thought necessary to approach this problem from three different angles:

1. A detailed study of sex-differentiation in males and females.
2. Isolation of a large number of females for sex observation.
3. An attempt to control sex experimentally.

Only the first part is considered in the present paper. The last two are still in progress and will be the subject of a future publication.

II. MATERIAL AND METHODS.

No attempt was made to study embryonic development during gestation, except for a few later stages, which will be described further on. Development during gestation seems to present some interesting features which will be further investigated as soon as the opportunity presents itself. My material includes fishes from birth to adult stage. To secure all possible stages in sex-differentiation, a large number of fishes were necessary; not less than 400 specimens were studied, of which 300 were sectioned for cytological study.

As to fixatives, Allen's, Bouin's, Child's, Flemming's strong, Smith's, and Zenker's fluids have been used, Bouin's giving constant and satisfactory results. For general stains Heidenhain's hæmotoxylin and alum-cochineal counterstained with orange G

were used successfully. For special stains Mallory's triple stain and Harris' hæmotoyxlin were used. All material except adult ovaries were sectioned in paraffin, the latter in celloidin. Very young fishes were sectioned as a whole, in medium-sized fishes the entire viscera were sectioned, and in the mature fishes the gonads and ducts only were preserved for sectioning. For general study sections were cut $6\,\mu$ in thickness.

The body form of the female is strikingly different from that of the male, which fact can be used to advantage in following the development of the species. To express it in numbers, the length of the fish is divided by its greatest depth. The resulting ratio is known as the form index and can be obtained in two ways, by using either the total length or the body length of the fish as the numerator. The former method is less subject to error in the case of *Xiphophorus helleri* and will be used exclusively in this work.

As the anal fin undergoes a marked post embryonal change in the males in which the third ray increases immensely in diameter, while the fourth and fifth rays, although participating in the transformation, suffer very little, if any, increase in diameter, a ratio of the third by the fourth ray suggests itself. This ratio may be called the fin ratio.

. It gives me great pleasure to acknowledge my indebtedness to Prof. F. R. Lillie and Dr. A. W. Bellamy for numerous suggestions and criticisms during the progress of this work. I also wish to express my appreciation for the most painstaking services rendered by our artist, Mr. K. Toda, our technician, Miss D. Brockett, and our librarian, Miss E. L. Dickinson.

III. THE INDIFFERENT STAGE.

At the time the young fishes are born they measure on the average 8 mm. in total length, while sex is unmistakably established at the length of 10 mm. This means that the indifferent stage of sex development is almost entirely pre-natal. The form index is 6.26 and the fin ratio is 1.0 at birth. The gonads, although small, are distinctly set off from the surrounding tissues and lie one on each side of the body cavity, immediately below the air bladder, suspended in a peritoneal sac. The peritoneal sac, which becomes the permanent wall of the gonad, is a protruded portion of the

lining of the body cavity.   The gonad inside of this sac consists of two kinds of cells:

1. Primordial germ cells.

2. Very much smaller cells with elongated nuclei, which can not be distinguished from the peritoneal cells and which tend to surround the germ cells to form follicles (Fig. 1).

The primordial germ cells are unmistakable.   They are the largest cells in the body of the fish and measure on the average, cell 14.4 μ, nucleus 8.4 μ, in diameter.   The nucleus takes a lighter stain than the cytoplasma and possesses one more or less distinct nucleolus.   The chromatin occurs most commonly in strands or loops immediately beneath the nuclear membrane.   The germ cells may be single or in nests of two or more cells.

As stated above, the pre-natal developmental changes have not been studied systematically.   Several stages, however, are on hand. The gonads assume the bilateral position at about 3.2 mm. in total length.   Before that the germ cells are in a single mass placed medially in the body cavity.   The peritoneum already surrounds the gonad more or less loosely and here the mingling of the peritoneal cells with the germ cells is very evident (Fig. 3).   As these rather small insignificant cells, mingling with the primordial germ cells, play by no means an insignificant rôle in later differentiation, I have been particularly anxious to trace their origin, and must conclude that no criterion whatever separates them from peritoneal cells.   This view is consonant with that of McLeod (1881), Jungerson (1889), and Eigenmann (1897).   The primordial germ cells of the 3.2 mm. stage are very conspicuous structures.   The cell is 11.8 μ and the nucleus is 6.4 μ in diameter.   The nucleus may be lobulated and appear as if more than one nucleus was present in a cell (Fig. 3).   The cytoplasm is yolk laden.   In short, the conditions of these cells indicate an early segregation.

IV.   Early Sex-differentiation of the Female.

The earliest stage of the ovary shows very little difference from the indifferent gonad.   In the early development of the ovary the primordial germ cells grow in size and form follicles.   At the time these follicles have reached approximately the medium size they degenerate and are absorbed.   This process is referred to as retro-

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gression. Definitive germ cells originate from peritoneal deriva-
tives later and differentiate into functional follicles of the adult
ovary. All young females undergo retrogression, of which three
classes may be distinguished. In some, hereafter distinguished as
class 1, formation of definitive follicles begins before retrogression
is well advanced. These comprise about 50 per cent. of all young
females (see Table IV., p. 68). In others (class 2) no formation
of definitive follicles precedes complete degeneration of primordial
follicles, but indifferent germ cells are seen in the epithelium be-
fore degeneration is complete. In others, again (class 3), retro-
gression of primordial germ cells is complete before any germ cells
appear in the epithelium. In the first class there is no doubt that
development into functional females occurs; the later history of
the other two classes is considered beyond. The ovarian cavity
and the oviduct are formed comparatively early in the development
of the ovary.

1. Early Normal Ovary.

The total length of the young female varies from 9.3 to 16.9 mm.
The average form index is 5.02 and the fin ratio is 1.13.

The indifferent gonad passes into the ovary very gradually.
Paired and considerably apart at the beginning; the two ovaries
approach each other until they meet medially and fuse into one
gonad which is the normal condition of the adult ovary. The
external contour of the gonad is very regular and its component
cells, the primordial germ cells and the peritoneal cells, are evenly
distributed (Fig. 2). The gonad gradually increases in size owing
to the multiplication of its component cells, although no mitotic
figures have been discovered. This is true of all the material on
hand.

At the time the two gonads have approached each other the pic-
ture has changed considerably. The primordial germ cells increase
in size and become completely invested by peritoneal cells to form
a follicle of one layer of cells thick. The growth of the germ cells
is not simultaneous, some grow faster than others, but all the germ
cells present in the gonad are subject to a transformation to young
ova at this stage or the early phase of the next stage. Blood vessels
which are present in the mesentery enter the young ovary in the
form of capillaries.

The shape of the approaching gonads changes. Instead of being broader laterally they now deepen dorso-ventrally. Fusion of the two gonads take place anterio-posteriorly, not along the entire surface of contact, but on the dorsal and ventral margins only. The space between becomes the ovarian cavity (Figs. 4–5). It will be recalled that the external lining of the gonad is of peritoneal origin, which means that the epithelium of the ovarian cavity is of peritoneal origin. At first the ovarian cavity is a narrow slit with its larger axis running dorso-ventrally; very soon, however, this picture changes to a cross-shaped lumen produced by the invagination of the epithelium laterally. This form of the lumen persists for a considerable time in the young ovary, but ultimately becomes very much modified until in the adult ovary no trace of the original form remains (Fig. 27). That portion of the mesentery which comes to lie between the fusing gonads becomes absorbed. The dorsal portion, or mesovarium, attaches the ovary to the body wall dorsally, while the ventral portion connects the gonad with the rectum ventrally.

The formation of the oviduct is begun at the time the two gonads are in close proximity. By the time the gonads are completely fused the duct tissue extends from ovary to urogenital sinus with complete lumen at anterior and posterior ends, but none or only a beginning centrally. In fact, the lumen of the oviduct appears first in its posterior terminal, and only later, at the anterior, end. This, of course, would indicate that the duct formation proceeds from two primordia, one anterior and the other posterior, which is the case in *Xiphophorus helleri*. The anterior primordium is the posterior end of the ovary and the posterior is the lining of the body cavity at its most posterior end in the region of the urogenital sinus. Before there is any indication of duct formation anteriorly, the posterior primordium is actively proliferating cells which at first form a solid cord along the median portion of the peritoneal lining of the body cavity on the dorsal edge of the mesentery. This cord extends anteriorly and at the time a similar strand of cells is formed from the anterior primordium a lumen has appeared in the former posteriorly (Figs. 10–11). No liquefaction of cells has been noticed in the formation of the lumen.

The anterior cord of cells is a direct extension of the posterior

ventral portion of the ovary. As that part of the ovary has not as yet fused dorsally, the extending cord is V-shaped with the apex ventrally (Fig. 6). This portion of the duct grows posteriorly on the dorsal edge of the mesentery until it reaches and fuses with its posterior member. The mesovarium thickens and flattens above the posterior portion of the ovary and finally comes in contact with the open end of the V to fuse and thus form the lumen in the oviduct from the anterior primordium (Figs. 6–8).

That the duct starts to grow from two sources was first noticed in an abnormality in which the two growing tips overlapped with the urinary bladder between. In normal development the duct forms ventral to the urinary system, but in the case in question the anterior part of the duct had pushed above the urinary bladder, while that from the posterior end was in the normal path. The overlapping was for a considerable distance. This stimulated closer study, and it was found that at the time when cords are formed at both ends no traces of such exist centrally (Fig. 14), nothing but a single layer of peritoneal lining. Further it was ascertained that by far the larger part of the duct comes from the posterior source.

In the primordial germ cells of the indifferent stage there is only one nucleolus with irregular, generally spherical contour. It always stains with chromatin dyes. Later its contour becomes perfectly regular and it enlarges. Still later two nucleoli are usually found, though as many as six may occur. In the ova of medium size (.272 mm.) the nucleoli are conspicuous bodies and may measure $10\,\mu$ in diameter. A body similar to the nucleolus in stain and size was noticed in the cytoplasm. At first it was taken for a centrosome, but as it showed no structure whatever the first hypothesis had to be abandoned. Subsequently it was noticed that one of the two nucleoli moves toward the nuclear membrane and passes through it (Fig. 13). When it reaches the cytoplasm it does not stay there, but moves toward the follicular wall (Fig. 16). It is probable that the migrating nucleolus passes out of the follicle, as it can be seen in all parts of the cytoplasm. The actual process of passing through the follicular wall has not been noticed, but bodies which answer to the description of the nucleolus are occasionally encountered between the follicles. Such migrating

nucleoli are by no means uncommon, as many as six have been noticed in one young ovary. The direction of migration is not predetermined. In retrogressive ova its shape is greatly affected. It may be elongated, crescent-shaped, irregular, or hollow.

2. *Retrogression in Class 1.*

The size limits of the fish vary from 16.5 to 29.5 mm. in total length. The form index averages 4.62 and the fin ratio is 1.16. The germ cells all derived from primordial ova in this stage are anywhere in size between primordial germ cells and medium-sized ova. However, the larger-sized ova are affected first. The first perceivable sign of retrogression appears to be a darker staining zone in the cytoplasm surrounding the nucleus. Later this zone gradually disappears, but at the same time the cytoplasm loses its affinity for stain and appears bleached. The follicular wall, which is one layer of cells thick in the normal ova, now becomes disorganized and it may form two or more layers of cells which may form strands and migrate into the yolk (Fig. 15). The nucleus appears to be affected last. It shrinks, becomes irregular, and disintegrates together with the yolk mass. After the ovum has been entirely absorbed remnants of the follicle remaining for some time witness to the destruction. Some such atrophied follicles may contain several isolated cells which no doubt are the remains of the ingrowing follicular strands. Sooner or later these remnants are also resorbed (Fig. 25).

The retrogression is further characterized by the fact that the epithelium of the ovarian cavity shows no signs of being unfavorably affected. On the contrary it appears very active. It has been noticed, particularly when the ova are greatly reduced by disintegration, that new primordial germ cells originate in the proximity of the epithelium of the ovarian cavity. These could not be traced to cell division, nor could they be traced to preexisting smaller primordial germ cells; the source of these cells is the epithelium itself.

It appears probable that any cell in the epithelium is capable of transforming into a germ cell. All stages of such transformation are encountered in the epithelium as will become apparent from Figs. 17–24. The epithelial cells are small with relatively little

cytoplasm; the nucleus is elongated and may be more or less ir-
regular in contour. The chromatin is granulated and an ill-defined
nucleolus can occasionally be noticed. The nuclei take a dark stain
and contrast strikingly with the germ cell nuclei. The first sign
of such metamorphosis appears to be the activity of the chromatin
in which the granules become coarser, forming more or less into
lumps. This phenomenon is closely followed by the growth of
nucleus and cytoplasm. The elongated and irregular contour be-
comes regular and the stain loses its density. The final product of
such transformed epithelial cells can not in any way be distin-
guished from primordial germ cells (Figs. 1–2). As these cells
increase in size they passively move from the epithelium into the
cortex of the ovary to develop into follicles. Fully formed germ
cells do occur in the epithelium, which will be discussed later. The
transformation of epithelial cells into germ cells is by no means a
rarity. It is a common occurrence in all phases of ovarian activity
of the females belonging to class I. Occasionally a place in the
epithelium may be found where all stages of such transformation
occur in the same field of the microscope. Such a condition is
found in Fig. 12. At least six definite stages (a–f) are shown in
the figure which range from epithelial to completely formed germ
cells.

It will be remembered that the epithelium of the ovarian cavity
originates from the peritoneal lining of the fusing gonads, and that
no germ cells whatever enter into its composition at any time.

The formation of germ cells from epithelium of the ovarian
cavity is by no means confined to *Xiphophorus helleri* among the
teleosts. Similar observations have been made by Hoffmann
(1886) and Böhi (1904) on salmon, Wallace (1904) on *Zoarces
viviporus*, Philippi (1908) on *Glandichthys januarius, et al.*

After the transformation process of the epithelium cells into
germ cells was discovered, it became apparent that such transforma-
tion may occur at any place in the ovary directly from the free
cells of peritoneal origin (Fig. 15, *tpc*). The process is the same
in principle as described above.

3. *Retrogression in Class 2.*

The general appearance of females of this class is not materially different from class 1. The total length varies between 14.2 and 29.6 mm. The form index averages 4.53 and the fin ratio is 1.17. There are generally fewer primordial ova in the ovary than in the previous class and hardly any one of them appear normal. The most important characteristic of this class lies in the fact that the epithelium of the ovarian cavity is relatively inactive.

The epithelium of the ovarian cavity is no longer surrounded by germ cells, nor are there many at any other place. Even the stroma is very sparingly represented. The ovary looks empty. The outer ovarian epithelium, although intact, shows similar signs of inactivity. Blood vessels are the most prominent structures in the ovary in this class of disintegration (Fig. 26).

4. *Retrogression in Class 3.*

The size limits in this class vary from 18.4–65.0 mm. in total length. The average form index is 4.43 and the fin ratio is 1.84. From Table I. it will be seen that the form index of this class is much closer to that of the male than to that of the female, and that the fin ratio is decidedly that of a young male. In fact, in some of the specimens in this class the anal fin has advanced considerably in the process of transformation. These facts are evidently significant and they will be referred to in connection with the differentiation of the male sex.

Conditions are not less significant as regards the ovary. The outer epithelium of the ovary has been either completely resorbed or else is in advanced stages of resorption. Ova of all stages have left nothing but inconspicuous traces of former presence. Nothing but the epithelium of the ovarian cavity and the oviduct has withstood the destructive process (Fig. 28). The epithelium has shrunk considerably and is by no means of the same appearance in all cases. It looks inactive in some and active in others, although proliferation of germ cells is not apparent. Judging from the appearance of the ovarian remains, it can be said that the renewed activity of the epithelium in later stages of retrogression is not simultaneous with degeneration, but begins afterwards. Remains

of degenerating follicles may be found in the body cavity. Occasionally such remains may assume activity and form cords of various sizes, which may persist for a considerable time.

The fatty tissue which commonly surrounds the gonads in fishes increases immensely during the retrogression. In fact, in the later phases of disintegration of the ovary special care must be taken to discover the remains of the ovary within the fatty mass. The fact that it contains an abundance of large blood vessels leads one to suspect that it might play some rôle in the disintegration process. Although stroma cells have been noticed among the fatty cells, at no time do the latter engulf, surround, or even come in contact with the disintegrating ova. Whatever physiological rôle they play as regards the fate of the germ cells can not be discovered by observation. Experimentally it has been noticed that the fatty tissue increases immensely within two days after injury to the reproductive system.

TABLE I.

Stages in Development.	Averaged Data.		Limits.
	Form Index.	Fin Ratio.	Total Length of Fish in Mm.
Indifferent	'6.26	1 0	0 - 9.3
Normal Immature Females	5.02	1.13	9.3 - 16.9
Retrogression in Class 1	4.62	1.16	16.9 - 29.5
Retrogression in Class 2	4.53	1.17	14.2 - 29.6
Retrogression in Class 3	4.43	1.84	18.4 - 65.0
Normal Mature Females. .	5.34	1.28	26.7 - 80.0
Early Tubule Formation.	5.17	1.16	9.3 - 13.5
Middle " "	4.84	1.27	12.5 - 18.6
Late " "	4.64	1.74	15.7 - 51.5
Mature Males . . .	4.20	4.25	31.6 - 84.4

V. EARLY SEX-DIFFERENTIATION OF THE MALE.

The testis of *Xiphophorus helleri* belongs to the acinus type in which seminiferous tubule formation is greatly modified. Sex-cords and tubules are formed from peritoneal cells exclusively. Such tubules give rise to the acini by transformation of epithelial cells into germ cells. The early sex-differentiation of the male is principally a process of tubule formation, and thus it may be divided into early, middle, and late stages of tubule formation.

1. *Early Stage of Tubule Formation.*

The size limits of the young male vary from 9.3 to 13.5 mm. in total length. The average form index is 5.17 and the fin ratio is 1.16, which differs very little, if any, from that of the female in similar stage of development.

The gonads, like those of the female, are far apart at the beginning of sex-differentiation. Gradual approach follows until they meet medially below the air bladder to be separated only by the mesentery. Unlike the ovary, the testes remain permanently paired. The mesentery is well supplied with blood vessels, which at this stage supply the gonads also. There is no difficulty whatever in distinguishing the testis from the indifferent gonad even in early phases of sex-differentiation. It will be remembered that such difficulty does exist in the case of the female. A comparison of Figs. 1 and 2 will make this clear. Even in much earlier stages (Fig. 3) this similarity exists. It may be that the indifferent stage is an early female stage.

Although I have not counted the germ cells in various stages of development, it is nevertheless apparent that the number of the primordial germ cells in the young testis is appreciably less than in the young ovary. Germ cell division was not seen and the cells usually remain isolated. The important rôle in the differentiation of the male is not played by the primordial germ cells, but by the peritoneal cells. The latter proliferate abundantly. and instead of being evenly mingled with the primordial germ cells, cause a segregation of the latter at the periphery of the testis, while the peritoneal cells occupy the center and inner margin (Fig. 30). This aggregation may be known as the sex-cord and, as will be seen in the next stage, is a preparation for tubule formation. In this stage also some of the peritoneal cells are investing the primordial germ cells. Such activity would indicate follicle formation, which is not an adult male structure.

2. *Middle Stage of Tubule Formation.*

The size limits of animals in this stage vary from 12.5 to 18.6 mm. in total length. The form index is 4.84 and the fin ratio is 1.27.

The primordial germ cells play no perceptible rôle in this stage.

They remain on the outer periphery of the gonads, apparently in-
active. All activity is vested in the cells derived from the peri-
toneum. These cells form at first a solid cord, the sex-cord, in the
central part of each gonad, parallel with the longer axis of the
animal. The cord formation in the young testis is antero-poste-
rior, but as the elongation of the gonad is mostly anteriorly, the
cord formation later follows the elongation of the testis. The cord
resembles very much a tubular gland except that there is no lumen
at first. The peritoneal cells place themselves side by side with
their apices pointing centrally in the sex-cord, which in a trans-
verse section appears rosette-like (Fig. 31). These cells increase
considerably in size and number, which causes the increase in diam-
eter of the cord and the origin of a central lumen; or, in other
words, transforms the cord into a tube which is the testicular cavity
or sperm duct. It must be pointed out that there is no indication
whatever that any of the germ cells participate in the formation of
the sperm duct.

3. *Late Stage of Tubule Formation.*

The size limits of animals in this stage vary from 15.7 to 51.5
mm. in total length. The wide range is significant in that it shows
great variation in the time of sex-differentiation in the male. The
average form index of this period is 4.66 and the fin ratio is 1.74,
which is decidedly male. There is a slight increase in diameter in
the third ray in the preceding stage, but, strictly speaking, trans-
formation of the anal fin into an intromittent organ or gonopod
does not begin before the present stage is reached. The gonad is
decidedly differentiated as testis before the anal fin is affected.
The first indication of the transformation of the anal fin into a
gonopod is the thickening of the third ray. This is shortly fol-
lowed by the elongation of the third, fourth, and fifth rays until
approximately twice the length of the original fin is reached. The
rest of the rays—*i.e.,* the first, second, and seventh to eleventh—
are not subject to any particular change.   .

The gonad has increased appreciably in size owing primarily to
the proliferation of the peritoneal derivatives and only secondarily
to increase of the primordial germ cells. Most commonly the
testis is butterfly-shaped in the transverse section, but may be very

irregular, depending upon its previous history. The primordial germ cells are almost invariably in nests, which would indicate that cell division has taken place; mitotic figures, however, have not been seen. These nests of germ cells always occupy the periphery of the testis and have no connection with the tubules.

The sperm duct or the main tube of the testis of the previous stage is now branched and as development proceeds the branches become subdivided (Figs. 32 and 38–39). Each testis may be roughly compared to a bunch of grapes with the sperm duct as the main stem. The tubules thus produced are radially arranged with their apices toward the periphery of the gonads. This tubule formation is peculiar to *Xiphophorus helleri* and closely related genera and, as far as the writer knows, has not been described before.

If one of these radial tubules is examined, it will be found that it consists of an inner epithelial layer and an outer homogeneous membrana propria (Fig. 33). This membrane is very thin and contains a very few small nuclei. The epithelial cells are cuboidal to columnar in shape, according to stage of development. In size and staining reaction the cells are intermediate between peritoneal and primordial germ cells. In more advanced stages of development the tubules show various stages of germ cell metamorphosis (Figs. 33–36). The apex may become separated from the rest of the tubule by a constriction and assume a spherical form; the tubular lumen has become obliterated by the growth of cells which are now spherical in shape and differ from the primordial germ cells at the periphery of the testis only in position. The next portion of the tubule shows cells in a less advanced stage; the lumen may still be present, the cells and particularly the nuclei are smaller in size and less regular in shape; the cells take a darker stain than the apical cells and there is no delimiting membrane from the more basal cells. The proximal portion of the tubule may differ in no essentials from the young tubule except that the cells show an increase in size. Later on the acini separate completely from the tubule, but for a long time the radial arrangement is maintained (Figs. 37–38).

It will be remembered that in the ovary definitive germ cells are formed from two different sources:

1. The epithelium of the ovarian cavity.

2. Free cells in the ovarian cortex of peritoneal origin. Similar conditions are found in the testis. The first and by far the most common source is the epithelium of the tubules described above. The second source, precisely as in the female, is the free peritoneal cells of the testicle. The method of transformation differs in no essentials from that found in the ovary. Such germ cells move toward the periphery of the gonad, multiply, and eventually become spermatocysts.

The variability in the development of the testis in this stage can not be overlooked. They occur too often and too regularly to be classified as haphazard abnormalities. The testis of the young fish from 15-20 mm. in total length is a very definite structure. Its definite shape, its regular contour, and its distinct bilaterality are characteristic (Fig. 38). In cases where transformation occurs, however, the gonad varies in shape, is irregular in contour, and, what is more striking, is only partially bilateral. Generally the posterior part of the testis shows more or less complete bilaterality, while the anterior part is in a fused state, thus producing a bifurcated testis (Figs. 40 and 41). There is no indication whatever that the typical shape of the testis occurring in those specimens that transform early becomes irregular and the bilateral testis becomes fused as development advances. On the contrary, the typical testis maintains its definite shape during the life of the animal, while the irregularly shaped testis gradually becomes regular as development proceeds, and at the time of spermatogenesis the shape of the testis is more or less definite in all. All of the above facts lead to the inference that the bifurcated testis has resulted from the epithelium of the ovarian cavity after complete disintegration of the ovules. In fact, all stages can be found between the epithelium of a degenerated ovary and a testis.

The formation of the extra-testicular sperm duct belongs to this stage, although some traces of it are already found in early and middle stages of tubule formation. Generally speaking, the male and female ducts are homologous. Both begin·from an anterior and a posterior primordium. In both the posterior primordium is more advanced in origin and extent of formation than the anterior primordium. In both the anterior portion of the duct is of peritoneal origin, and this is in all probability true of the posterior por-

tion as well.   The formation of the lumen of the posterior part of
the duct is the same in both sexes.   The anterior portion, however,
presents some slight differences.   In the male the lumen is formed
by the extension of the intra-testicular sperm duct into the poste-
rior primordium.   The ends of the two sperm ducts meet slightly
back of the testis and fuse to form a single duct.   Not only is the
origin and method of formation of the male and female ducts simi-
lar, but the young sperm duct and the young oviduct are identical
in structure.   Specialization takes place only later in development.

VI.   LATE SEX-DIFFERENTIATION OF THE FEMALE.

The smallest normal mature female found in my material is
26.7 mm. in total length.   The largest is 80.0 mm. long, which can
be considered a full-grown specimen.   The average form index is
5.34 and the fin ratio is 1.28.

Externally the sexually mature female can be recognized by the
appearance of a dark spot on each side in the region of the pelvic
fins.   This is not due to pigmentation of the dermis, but of the
peritoneal lining of the body cavity above and posterior to the
ovary.   In males and young females the peritoneal lining is silvery
white or slightly pigmented.   As the female approaches sexual
maturity the pigment increases immensely and can be seen through
the translucent body wall as a black spot.   The name " Trächtig-
keitsfleck " has been applied to denote this characteristic, but since
it is maturity instead of " Trachtigkeit " that causes its appearance,
a more appropriate name would be puberty spot.   The size of the
spot varies directly with the size of the ovary and the period of
gestation.   It is very conspicuous at the time of the birth of the
young and this probably explains the origin of the German name.

A female with a puberty spot always contains mature ova.
There may be from one to nearly one hundred in number, depend-
ing upon the age of the fish.   They are of a brilliant amber color,
measuring on the average 1.6 mm. in diameter.   The ova lie in the
cortex of the ovary and often occupy the entire space between
ovarian lumen and ovarian wall (Fig. 27).   Each is contained in
a follicle consisting of a single layer of cuboidal cells.   The fol-
licles are surrounded by a very slightly developed theca folliculi
which emerges indistinctly into the surrounding stroma.   Imma-

ture ova of all sizes can be found all over the ovary, but most commonly in the region of the epithelium of the ovarian cavity. Yolk is deposited at the time the ova have reached medium size. At this time the young egg has a beautiful alveolar structure in section due to the presence of oil globules which have been dissolved by reagents.

The epithelium of the ovarian cavity of the adult *Xiphophorus helleri* differs in no essentials from that of *Glaridichthys* described by Philippi (1908). Owing to the encroachment of the adult ova it has been thrown into numerous folds which occasionally are not unlike the villi of the small intestine. The characteristic depressions in the epithelium of the ovarian cavity called "Delle" by Stuhlmann (1887) are very prominent in *Xiphophorus helleri*. Each depression is an invagination of the epithelium of the ovarian cavity into the ovarian substance directed toward an egg. It serves two purposes: admission of the sperm into the ovum and creation of a place of rupture for the escaping young. Its formation begins with rather young ova, slightly below medium size, but is not completed until the time of fertilization.

The oviduct of the adult female is very short. It consists of three layers: an outer muscular, a middle connective tissue, and an inner epithelial. The latter is thrown into folds which may either project into the lumen like villi or overlap and form pockets. According to Philippi (1908) these pockets serve as hiding places for spermatozoa in which they may remain and maintain vitality for over 160 days. The oviduct enters the uro-genital sinus at its anterior border, projects backward to open immediately in front of the aperture which is located slightly back of the anus.

VII. LATE SEX-DIFFERENTIATION OF THE MALE.

Here are comprised the stages in which spermatogenesis takes place. The size limits vary from 30 to 84.4 mm. in total length. The form index of the sexually mature male averages about 4.2, while the fin ratio is 4.25.

The various stages of spermatogenesis are rather difficult to describe owing to the fact that cell division has thus far evaded the writer's observation. Of course, the difficulty lies mostly in the proper application of terminology and not so much in the changes that take place.

As has been pointed out, the acinus or spermatocyst is the end product of the tubule formation and the beginning of spermatogenesis. The definitive germ cells in the acini approach the size and appearance of the primordial germ cells before spermatogenesis begins. At this period the nuclei of these cells measure from 7.2 to 9 μ in diameter. This is the largest size they ever reach. The cell dimensions are hard to measure because the entire acinus presents the aspect of a syncytium (Fig. 36). On account of the size and what is to follow, it would seem proper to term these cells primary spermatocytes. The next thing that happens to the acini is the doubling of their volume and the number of their cells. Evidently cell division has taken place. The picture has changed radically (Fig. 37, ssc). The nuclei no longer show a clear aspect with more or less thread-like chromatin; the stain is darker, the chromatin is broken up into a great many rod-like structures; the nuclear membrane is absent and the cytoplasm is greatly diminished. The nuclei now measure on the average 5.96 μ in diameter. This might be considered a secondary spermatocyte stage.

Apparently another division takes place as the number of nuclei doubles. This time there is no appreciable increase in size of the acini. The size of the nucleus, however, has diminished to 2.5 μ, which is practically one half of the previous stage. The color has not changed, but the cytoplasm is reduced to a minimum; in fact, it can hardly be demonstrated with general methods of staining (Fig. 37, sds). It is probable that we are dealing with the spermatid stage.

In the next stage the metamorphosis of the spermatids into spermatozoa is quite evident. For the first time the acinus becomes luminated as the developing spermatozoa move toward the very thin, homogeneous wall of the acinus, where they form a complete layer of one layer of cells thick. The tail differentiates and the spermatozoa are all oriented so that the tail is free in the lumen. The head, or rather the nucleus, gradually decreases in size as it elongates. The final dimensions are 1.35 x 2.55. There is also a decrease of size in the entire acinus or spermatophore, which measures about 50 μ in diameter, or half that of the secondary spermatocyte stage. The heads of the spermatozoa are so closely pressed together that they appear like a single layer of epithelial cells (Fig.

37, *sph*). They stain very dark and with iron-hemotoxylin the cross-section of a spermatophore presents a solid dark ring. The tails are fairly long and are twisted altogether in a sort of spiral. Occasionally a belated spermatozoan has failed to get "in line" and remains in the lumen among the tails. The ripe spermatophores occupy the central portion of the testis and are found in every part of the sperm duct. In this condition they are discharged and reach the female genital tract, where by the action of the ovarian secretion the outer membrane is dissolved and the spermatozoa swim freely in the oviduct or ovarian cavity.

The question naturally arises as to the meaning of the primordial germ cells at the periphery of the testis (Figs. 37–38, *ppgc*). In comparison with the rest of the germ cells, they appear inactive. It can not be said with certainty that their number decreases with age, nor is there any reliable sign that they take part in spermatogenesis. In a few diseased males which were affected by a tumor growth in the tail region the testis was affected to a marked degree. All the spermatophores, mature and immature, were broken up with the spermatozoa free in the lumen. Formation of new acini was going on in all parts of the testis, but the peripheral primordial germ cells showed no more activity than in the normal. This seems to lend evidence that the primordial germ cells do not furnish a source of definitive germ cells.

It seems to be necessary to emphasize at this place that no retrogressive development of any sort is encountered in the normal process of sex-differentiation in the male of *Xiphophorus helleri*.

The sperm duct is slightly longer than the oviduct because the testis is more anterior in position. It consists of three layers: an outer muscular, a middle connective tissue, and an inner epithelial. The cells of the epithelium are flagellated. The contour of the epithelium is very regular. Just as in the case of the female, the sperm duct enters into the uro-genital sinus to open just in front of the aperture. In both male and female the urethra opens into the anterior part of the uro-genital sinus. The uro-genital aperture lies at the base of the gonopod and does not enter into it.

It will be remembered that the transformation of the anal fin into an intromittent organ or gonopod begins during the late stage of tubule formation. The first noticeable thing in such meta-

morphosis is the thickening of the thírd ray; in fact, it should be said rays, as all of them are paired and lie side by side (Figs. 44 and 45). As the thickening of this ray continues, the third, fourth, and fifth rays elongate until approximately twice the length of the original fin is reached (Figs. 48 and 49). Next the tips of the last-named rays form knob-like projections which are to be transformed into hooks in later development (Fig. 47). As a general rule, spermatogenesis begins at this stage of the transformation of the anal fin, although variations are not uncommon. The first, second, and sixth to tenth rays are subject to no special changes and remain rudimentary. The third ray, which has increased immensely in thickness medially, forms an S-like structure apically and the distal arm projects out on the ventral margin of the fin to form a copulatory hook. This hook is reinforced by the tip of one of the members of the fourth rays, which has turned ventrally, and we shall call it the ventral part of the fourth ray. The same kind of a hook is formed on the opposite margin of the fin by the union of the two members of the fifth ray (Fig. 46). Furthermore, the third and the dorsal part of the fourth ray form two rows of symmetrically placed "teeth" which project backwards and outwards, forming a hollow external groove in the former which leads toward the tip of the gonopod. There are approximately 8 " teeth " on each side of the third ray and 9 in the fourth. The two members of the fifth ray in the secondary growth regions, anterior to the hook, have fused and broadened latero-dorsally to form a concave groove on the dorsal margin of the gonopod. All the apical modifications of the rays are supposed to be for copulation and transmission of the spermatophores from the male to the female. At the time the anal fin is metamorphosed into a gonopod the spermatophores are formed and ready for discharge.

Besides the gonopod the beautiful sword of the *helleri* male and the pelvic fins are secondary sex characters and must be considered here. Both of them, however, start their transformation somewhat later than the anal fins.

The sword shows the first signs of development shortly before spermatogenesis begins. It is formed from the ventral lobe of the tail fin of the male fish. The ten ventral rays of the caudal fin are involved; most of the elongation falls upon the sixth to

5

tenth and the utmost length is reached by the eighth ray. In the adult male the length of the sword approximates the total length of the fish. The pigmentation of the sword is very striking. The middle rays are from greenish to orange in color; this is bounded on both sides by deep black. The dorsal portion of the caudal fin has a yellowish hue.

The pelvic fins of the male differ from those of the female in the relative length of the rays. The length of the first or anterior ray of the pelvic fin of the male is to the second ray as 1 : 2, while the reverse is true of the female—*i.e.*, as 2 : 1. These differences appear late in sex-differentiation, approximately at the beginning of spermatogenesis.

VIII. SEX-RATIOS.

The sex-ratio in the immature fishes between the sizes of 10–26 mm. in total length is given in Table II. These limits were chosen because sex is unmistakably established at 10 mm. and normal adult females appear at about 27 mm. in total length. The specimens in this table were taken at random and thus they present the actual conditions in the population.

TABLE II.

Normal Females.		Retrogressive Females.		Males.		Total.		Percentage.	
No	Size	No.	Size.	No.	Size.	Males	Fe-males.	Males	Fe-males.
21	10 – 16.7	58	12.5 – 26	44	10 – 26	44	79	36%	74%

It is very clear from the above table that there is a great preponderance of females in *Xiphophorus helleri* in the immature condition—*i.e.*, in the developmental stages falling between 10 and 25 mm. in total length. For all practical purposes the ratio may be reduced to two females to one male.

For sex-ratios in mature fishes the writer is indebted to Dr. A. W. Bellamy. Table III. is compiled from his data. Each record gives the history of an entire brood reared from birth to sexual maturity.

TABLE III.

Record.	No. Born.	Females.	Males.	Unac-counted.	Percent. of Males.
106 E...	40	1	35	4	94
114 A....... .	13	2	11	0	85
106 D....... .	81	0	60	21	100
112 DEF..... .	106	26	33	47	56
112 G	47	27	6	14	18
34 AB.	12	1	9	2	90
113 ABC. . . .	17	2	10	5	83
Total. . ..	316	59	164	93	75 %

From the above tables it is evident that the sex-ratios have suffered reversal from immature to mature conditions; also that the change is very marked. One can perceive that such change could be brought about in two ways: differential viability and sex inversion.

Oxygen consumption experiments have shown (Bellamy, 1922) that the males consume approximately twice as much oxygen as the females. The males are also by far the more active. From these data one might expect to find greater mortality among the males than among the females. This is borne out experimentally. If both sexes of equal chronological age are subject to unfavorable conditions such as weak solutions of potassium cyanide, alcohol, or excesses of temperature, the male invariably succumbs first. With some of these conditions, such as cyanide or alcohol, the females live twice as long on the average as the males and sometimes even longer. That the reversal of the sex-ratio is not due to mortality of the females can also be ascertained by keeping strict records of broods of fishes from birth to sexual maturity. The mortality of Xiphophorus helleri under proper conditions is slight, indeed, and whenever death occurs the sex can be established cytologically. The results of such observations are decidedly in consonance with the experimental results and it is perfectly safe to conclude that the female is at least as sturdy as the male.

If the reversal of sex-ratios from immature to mature condition is not due to differential viability, there is only one other possibility, and that is sex inversion. This occurs most commonly in fishes from 16–27 mm. in total length, but may occur at any size from 16 mm. upward.

IX. Inversion of Females.

It will be seen in Table II. that all females between 16.7 and 26 mm. are retrogressive. Normal females do not occur before they reach approximately 27 mm. in total length. The phenomenon of retrogression in *Xiphophorus helleri* is not a seasonal fluctuation, as this species produces young practically every month. Nor is it temporary and reversible, once the ova are affected they can not be rejuvenated. Lane (1909) has described retrogressive ovules in *Lucifuga* and *Stygicola*. In these teleosts the larger and more favorably situated ova cause the disintegration of smaller ovules which are absorbed as food. It is very clear that this is not the case in *Xiphophorus*, for it is the larger ova that are affected first. These may be in a hopeless state of degeneration before the smaller ones show signs of disintegration. My material strongly favors the conclusion that all of the linear descendants of the primordial germ cells disintegrate.

If the retrogressive females transform into males, it is apparent from the above tables on sex-ratios that approximately half of the females become males. It is doubtful whether any of the females of class 1 transform, because the ovarian epithelium is actively proliferating definitive germ cells. The activity of the epithelium is relatively slight in females of class 2 and stops entirely in females of class 3, and therefore one would expect that the prospective males are recruited from the two latter classes. If so, the sum of the females of classes 2 and 3 must approximately equal the sum of females of class 1. This is actually the case, as is evidenced by the following table, in which all of the retrogressive females studied are tabulated.

Table IV.

Class 1.	Class 2.	Class 3.
40	25	10

The question may be raised whether the retrogressive females result in sexual forms. In the first place, no sexless form has been encountered in the 300 fishes used for cytological work unless the females of class 3 be so considered. Careful study shows conclusively that they are not stationary but transitional forms, and

that they are in stages between a completely disintegrated ovary and the origin of the male gonad—*i.e.*, the late stage tubule formation. In the second place, breeding experiments have shown that there are no sterile individuals unless diseased, which, as I have pointed out, are not very common. There are several cases where a fish has been selected as a normal female and placed in an aquarium with a male for breeding purposes and after several months two well-formed males have been found in the aquarium.

Finally the evidence that such retrogressive females may develop into males may be summarized as follows:

1. The sex-ratios described above, in which the ratio is reversed at maturity from what it was in immature stages, favors very strongly the idea of sex-inversion.

2. The occurrence of a bifurcated testis in certain males (see p. 60) which is to be explained as a connecting link between female and male.

3. The irregular contour of the testis and the large and irregular tubules of the presumed arrhenoids (Figs. 40–41) contrast conspicuously with the normal testis (Fig. 38).

4. The condition of the anal fin is further supporting evidence. Making ample allowance for all errors, the average fin ratio of the retrogressive female is far into the domain of the male (Table I.). In fact, the general appearance of such an anal fin suggests the male condition in the stage of late tubule formation, whereas there is nothing more than the remains of the epithelium of the ovarian cavity.

5. The form index and the advanced age of the arrhenoid fish as compared with normal sex-differentiation of the male are additional arguments in favor of sex-inversion in *Xiphophorus helleri.*

The opinion of breeders and fish fanciers has been for a number of years in favor of sex-inversion in *Xiphophorus helleri* and related forms. Their observations have been confined naturally to external features; mainly to form index and anal fin. I have had the opportunity to meet the members of the Chicago Aquarium Club and hear reports on first-hand observations dealing with the problem. Several members of this club have favored me with material of various kinds for which I wish to express my sincere appreciation.

Several reports dealing with sex-reversal in *Xiphophorus helleri* have appeared in *Wochenschrift für Aquarien und Terrarien Kunde*. The following may be cited from No. 17, August, 1920, p. 273: " Eine längere Abhandlung bringt Herr A. Poser über die Umbildung von Weibchen in Männchen bei Helleri. Herr P. welcher ganz der Absicht des Herr Dr. Mertens ist (" Bl." 20, No. 13) wird von anwesenden Mitglieder der Erweis erbracht, dass auch bei uns diese Umbilding bei roten H. beobachted wurde. Eine Verwechlung mit anderen Jungtieren anderer Weibchen ist ausgeschlossen, da nur ein Exemplar vorhanden war. Die Umbildung vollzog sich sehr langsam."

X. COMPARATIVE.

It may be of interest to submit a brief comparative summary of some of the publications dealing with the·instability of sex in animals. This will be done under three headings: sex-inversion in teleosts, sex-inversion in other vertebrates, and the origin of definitive germ cells.

1. *Sex-inversion in Teleosts.*

As far as the writer has been able to ascertain the first report on arrhenoid fishes was furnished by Herzenstein (1891). The report concerned *Gymnocypris potanini* and *Schizopygopsis Güntheri*, both cyprinidont fishes. The observation was based on secondary sex characters—*i.e.*, on the assumption by the female of the secondary sex characters of the male.

Philippi (1904) reported " Ein neuer Fall von Arrhenoidie " in the viviporous teleost *Glaridichthys caudimaculatus,* which he describes as follows: " Ich isolirte anfangs October zwei anscheinend trächtige Weibchen von G. caudimaculatus zwecks besser Beobachtung. Während das eine an 17 October Junge warf, zeigte das andere drei oder vier Tage vor diesen Datum eine Veränderung an der Analflosse, die aber so schwach war, dass· ich über ihr Wesen nicht ins Klare kommen konte. Am 17 October war diese Veränderung so weit vorgeschritten, dass sie als schwache, aber deutliche Verlängerung der vorderen Strahlen erkennbar war. Am 7 November war die Analflosse bereits bis auf etwas das Dappelte des Normalen Ausgezogen, so dass die die eines halberwachsenen Männchen in der Form glich."

In 1908 the same author reported three more cases of arrhenoidy, all of which were *Glarydichthys januarius*. In all three the form index was strictly female, while the gonopod was well advanced. After one of them died it was examined microscopically and showed total absence of a gonad, while the duct was typically oviduct. It is clear that this particular fish was in a late retrogressive stage, when it is very difficult to isolate the epithelium of the ovarian cavity. In one of the remaining two Philippi found the following peculiarities: " Makroskopisch liess dieses 3, ausserlich in bezug and die grösse ganz als Weibschen erscheinende Tier 2 nicht miteinander verschmolzene milchweisse Hoden erkennen, in deren einem 2 dottergelbe grosse Eier sich befanden und die beide einem typischem Oviduct Aufsassen."

Newman (1908) reported on a case in *Fundulus majalis*, which he at the time called " A significant case of hermaphroditism in fish." As the specimen had advanced toward maleness in morphology and behavior during the period of observation, and as *F. majalis* is decidedly bi-sexual, the case is apparently one of sex-inversion.

2. Sex-inversion in Other Vertebrates.

The work by Brandt (1889) on birds is of considerable interest. The name "arrhenoidic" was coined to replace "Hahnenfedrigkeit." It deals mostly with the domestic fowl, but observations on game birds, etc., are not uncommon. Birds that have laid eggs and have otherwise appeared and acted like females have been observed to assume the appearance and behavior of the male sex. Such changes occur most commonly with senility, but this is not necessary, for birds of one year of age are known to transform. It has been noted that some abnormalities, such as a solid or blind duct, occur, but there are many with no such apparent causes. Cytologically various stages of disintegration of ovarian structures and new formation of apparently testicular tissues go hand in hand. The differentiation of seminiferous tubules first begins as solid cords which luminate and may develop from one to several layers of epithelium. Such tubules show signs of spermatogenesis; no spermatozoa, however, have been encountered.

Work of somewhat similar character has been described by Pearl

and Curtis (1909), Boring and Pearl (1918) on domestic fowl, and Pearl and Surface (1915) on the cow.

An interesting case of complete sex-inversion in Tritons was reported by Champy (1921). Here the inversion was from male to female, which is generally considered uncommon. The Triton in question was used for breeding purposes as a male. The transformed Triton had a typical oviduct and juvenile ovary containing numerous oöcytes which were in the process of yolk formation. Champy's conclusion is as follows: " Ensomme nous avons chez un animal adulte l'etat ovarien d'une femalle jeune. Etant Dannee l'histoire anterieure de l'animal, il n'est pas doutex que nous avons un cas d'interversion sexuelle totale."

Frogs and toads have long been known to show peculiarities as to sex conditions. Many of such abnormalities or " hermaphrodites" have been described by various observers. Lately Crew (1921) has shown that all of the " abnormalities which have been recorded can be so tabulated that the first case most nearly approximates to the normal female and the last the typical male, with respect to the nature of both primary and secondary sex characters. Thus arranged it is seen that the cases furnish an almost complete series of gradations which range from an individual almost completely female to one almost completely male, and that the conditions found readily appear to be merely graded stages of a single process."

Witschi ('21), after a thorough investigation of the sex conditions in frogs, concludes as follows: " Die Froshzwitter sind stets genetische Übergangsformen zwischen den reinen Geschlechtern (Übergangshermaphroditen), und zwar geht die Entwicklung von weiblichen zum männlichen Geschlecht."

3. Origin of Definitive Germ Cells.

It was pointed out in the foregoing pages that in the teleost a number of investigators have observed the origin of the definitive germ cells from peritoneal derivatives. Such conditions have been reported by Hoffmann (1886), Böhi (1904), Wallace (1904), and Philippi (1908).

Swingle (1921) reported that the lineal descendants of the primordial germ cells in *Rana catesbeiana* degenerate. As to the

origin of the definitive germ cells, he says: "In the interval between the first and second larval sexual cycle following the degeneration of large numbers of maturation cells the gonad becomes filled with small cells which, because of their size, nuclear structure, and staining capacity, appear as transition stages between mesothelial cells (germinal epithelium and sex-cord elements) and true germ cells. The later history shows them to be germ cells, but their origin is open to two interpretations and is not as clear as could be desired. The writer considers these cells as small germ cells descendants of the primordial sexual elements, and not as transformed germinal epithelium elements, but admits that the evidence from his material is equally strong for the germinal epithelium viewpoint."

Clearer cut results have been obtained by Firket (1920) in the albino rat. The primordial germ cells disintegrate and have disappeared entirely in the testis of the albino rat from the tenth to the fifteenth day after birth. The origin of the definitive germ cells in the albino rats, according to Firket, are as follows: "At the time the first spermatogonia appear they are easily recognizable by the texture of their nuclei and are very numerous. This has been shown very distinctly in the same species by Hoven. Let us insist that those spermatogonia can only be derived from the small epithelial cells, as they are at this stage the only type of cells present in sufficient numbers in the sex-cord. The spermatogonia must be called 'secondary germ cells.'"

It was stated previously (p. 47) that the data presented in this paper comprise only a part of the study of the sex problem of *Xiphophorus helleri.* Particular interest is attached to the experimental work in progress at the present time. Closer cytological study of the material is imperative to elucidate difficulties encountered in cell division, chromosome composition, etc. A discussion of the bearing of the present data on the theory of sex will be postponed until the completion of the entire problem.

XI. SUMMARY AND CONCLUSIONS.

1. At birth the young fish measure on the average 8 mm. in total length and are on the verge of sex-differentiation.

2. The gonads of the indifferent stage are paired and widely

apart, suspended in a peritoneal sac immediately below the air bladder.

3. The indifferent gonad consists of two kinds of cells: primordial germ cells and cells of peritoneal origin. Both are evenly distributed in the gonad.

4. At 10 mm. in length sexes are distinct. In females the indifferent gonad develops into an ovary without any marked morphological changes of the gonad except that the primordial germ cells gradually enlarge and become oöcytes.

5. In males distinct changes take place in which the germ cells and the peritoneal cells are segregated, the former on the periphery of the gonad, the latter occupy the median and inner portions of the gonad.

6. The paired gonad in the female fuses to form a single ovary. The median surface of the fusing gonads become the ovarian cavity.

7. In the male the gonads remain paired permanently.

8. Both the oviduct and the sperm duct are formed from two sources: posteriorly from the peritoneal lining of the body cavity and anteriorly from the gonad.

9. All females from about 12.5–26 mm. in total length are subject to retrogression. To all appearances all primordial germ cells 'disintegrate. Definitive germ cells come from peritoneal cells.

10. Completely disintegrated ovaries have been found with epithelium of the ovarian cavity appearing from very inactive to very active. The anal fin in such cases is in early stages of transformation into a gonopod.

11. The fate of the primordial germ cells in the male is uncertain. Definitive germ cells originate from peritoneal cells.

12. Bifurcated testes occur in a large number of cases, which are supposed to originate from a completely disintegrated ovary.

13. Sex-ratios are reversed from immature to mature condition.

14. Differential viability is in favor of the female—i.e., the female is at least as sturdy as the male.

15. The material decidedly favors sex-inversion in *Xiphophorus helleri.* This takes place most commonly in immature fishes, but may occur in adult animals.

16. The transformation of the anal fin into a gonopod takes

place after differentiation of the testis in normal males, but before differentiation in transformed males.

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EXPLANATION OF PLATES.

All the drawings have been made with the aid of an Abbe Camera. The lenses used were of Zeiss make and the magnifications follows the description of each figure.

PLATE I.

FIG. 1. Transverse section of indifferent gonad of a specimen 9 mm. in total length. × 1000.

FIG. 2. Transverse section of an early normal ovary. × 1000.

FIG. 3. Transverse section of an indifferent gonad of an embryo 3.2 mm. total length. × 1000.

Symbols :

- *bc* Binucleated germ cell.
- *ee* External epithelium of gonad.
- *ln* Labulated nucleus of germ cell.
- *m* Mesentery.
- *mo* Mesovarium.
- *p* Peritoneum.
- *pc* Peritoneal cell.
- *pgc* Primordial germ cell.
- *rc* Red blood cell.
- *yg* Yolk globules.
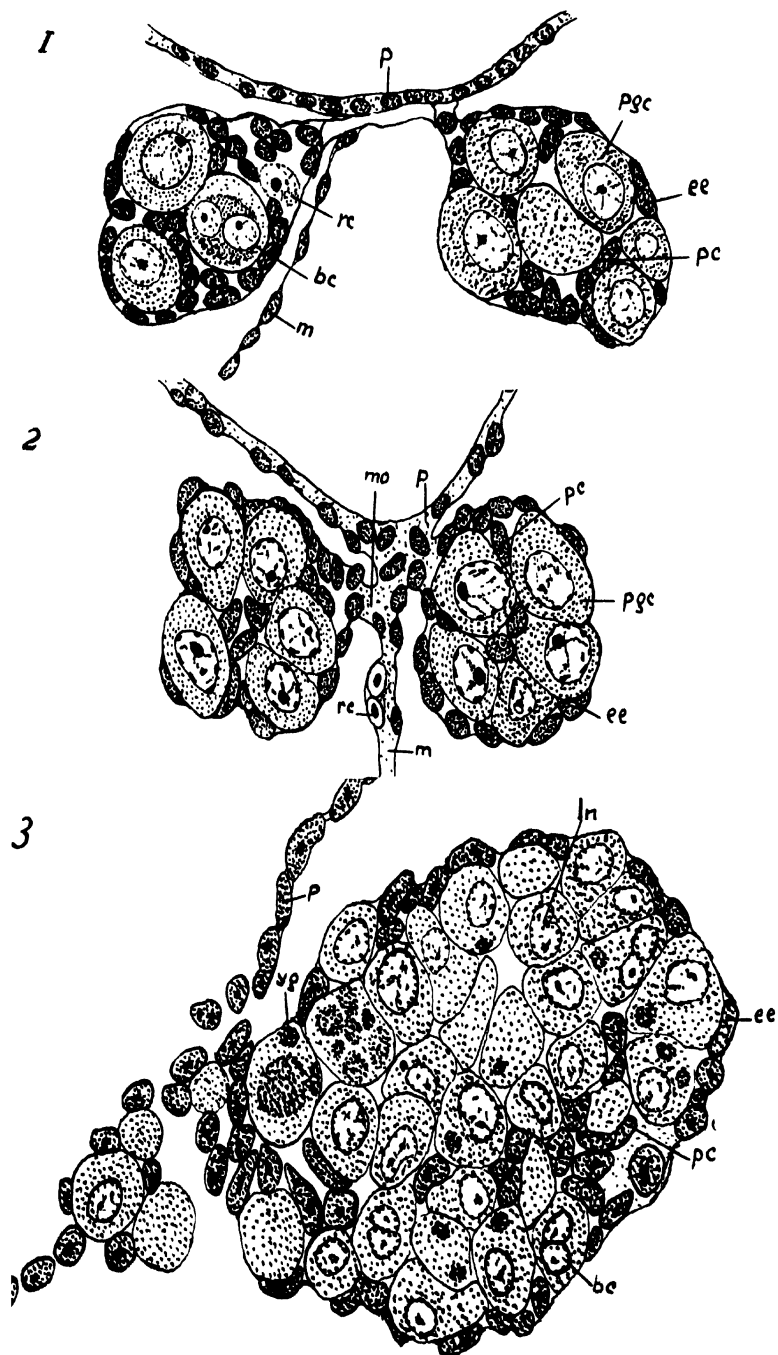
Note: All drawings of this plate are made from a preparation of a young normal ovary of a single individual.

FIGS. 4–5. Transverse section of anterior and posterior parts of ovary. × 115.

FIG. 6. Transverse section of the anterior primordium of oviduct through the anterior region. × 115.

FIG. 7. Transverse section of the anterior primordium of oviduct through middle region. × 115.

FIG. 8. Transverse section of the anterior primordium of oviduct through posterior region. × 115.

FIG. 9. Transverse section of central portion of oviduct showing a solid cord of cells. × 115.

FIG. 10. Transverse section of posterior primordium of the oviduct through the anterior region. × 115.

FIG. 11. Transverse section of posterior primordium of the oviduct through the posterior region. × 115.

Symbols:

ee External epithelium of gonad.
epo Epithelium of ovarian cavity.
ft Fatty tissue.
lo Lumen of oviduct.
m   Mesentery.
mo Mesovarium.
nt Nest of primordial germ cells.
o   Ovum.
oc Ovarian cavity.
od Oviduct.
p   Peritoneum.
pgc Primordial germ cells.
ppo Posteriorly extending portion of ovary.
rt Rectum.
thm Thickened portion of mesovarium.
ub Urinary bladder.
ur Ureter.
urt Urethra.
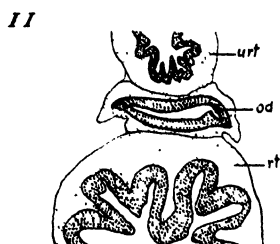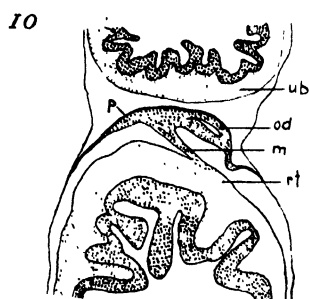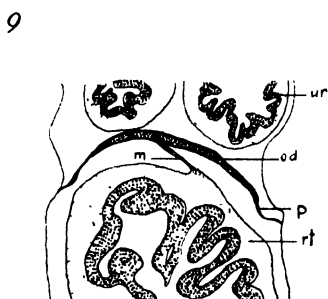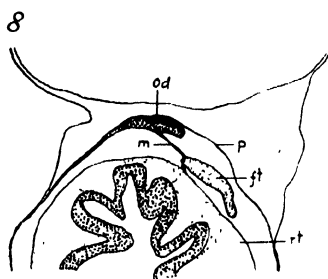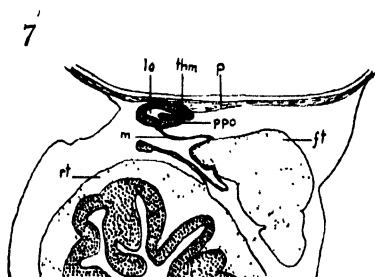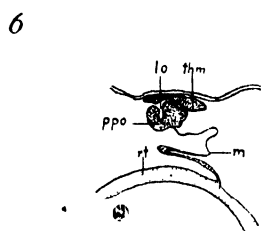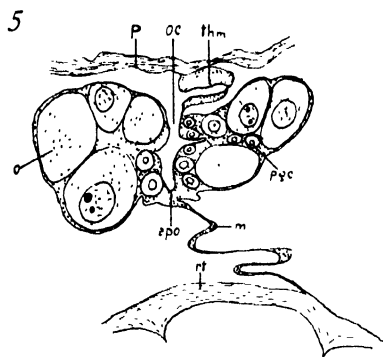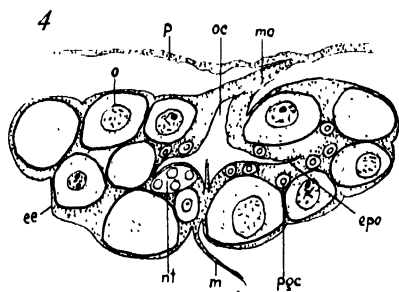
PLATE III.

Fig. 12. Portion of transverse section of epithelium of the ovarian cavity showing transformation of epithelial cells into definitive germ cells. × 1000.

Fig. 13. Section of medium-sized ovum to show migration of nucleolus from nucleus into cytoplasm. × 410.

Fig. 14. Portion of a transverse section of a young female between anterior and posterior primordia of oviduct showing no central duct formation. × 1000.

Fig. 15. Portion of a section of a disintegrating ovum showing disorganized follicular epithelium. × 1000.

Fig. 16. Section of medium-sized ovum to show migration of nucleolus towards periphery of ovum. × 416.

Symbols:

- *a–f* Various stages of transformation of epithelial cells into definitive germ cells.
- *bv* Blood vessel.
- *ec* Epithelial cell.
- *fe* Follicular epithelium.
- *m* Mesentery.
- *mno* Migrating nucleolus.
- *no* Nucleolus.
- *oc* Ovarian cavity.
- *p* Peritoneum.
- *rt* Rectum.
- *thf* Theca folliculi.
- *ub* Urinary bladder.
- *y* Yolk.
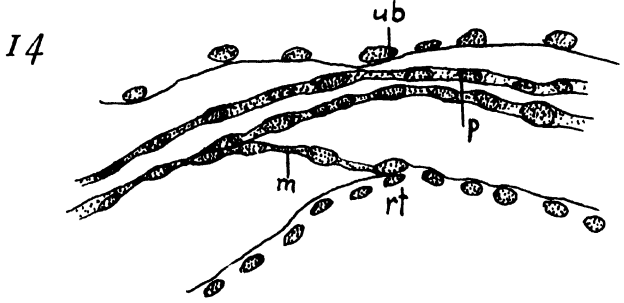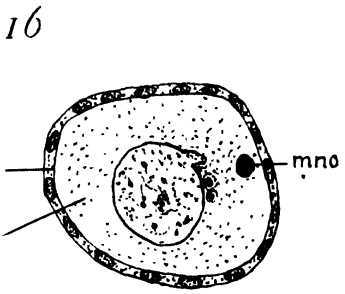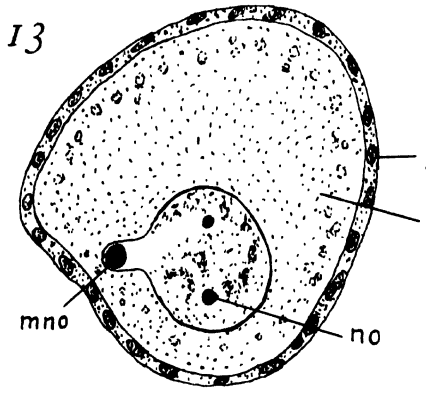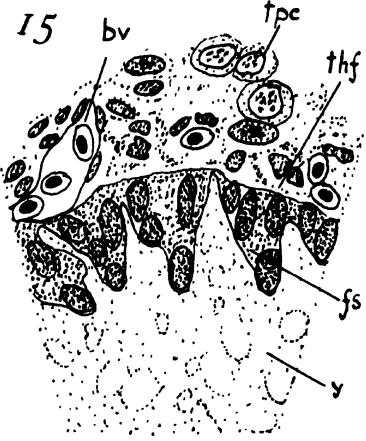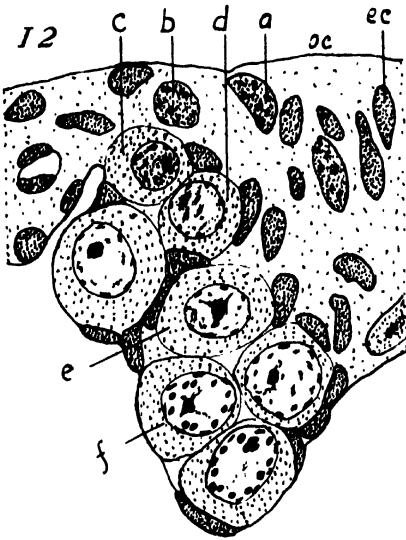
FIGS. 17–24.   Portions of transverse section of the epithelium of the ovarian cavity showing transformation of epithelial cells into definitive germ cells. × 1600.

Symbols:

   a–f Various stages of transformation of epithelial cells into definitive germ cells.

   oc Ovarian cavity.
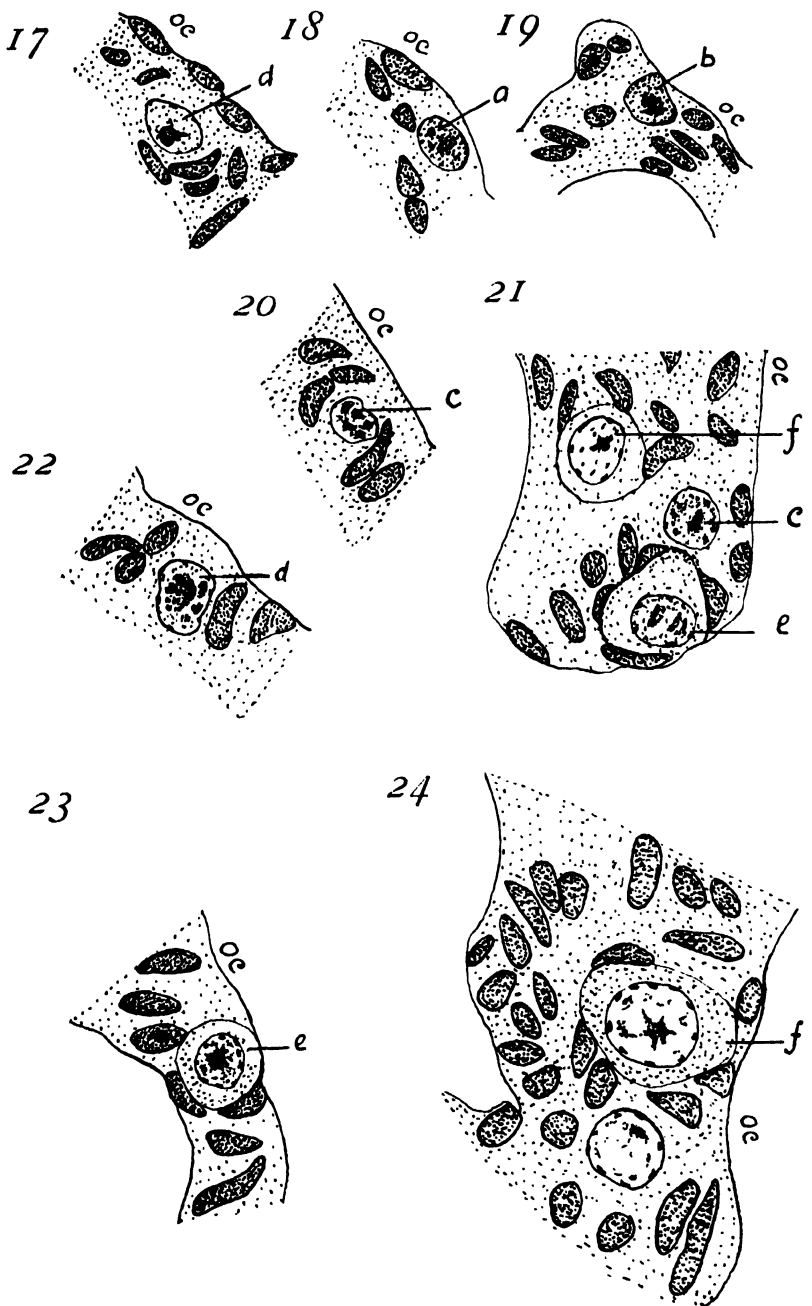
PLATE V.

FIG. 25. Transverse section of retrogressive ovary in class 1.   × 80.
FIG. 26. Transverse section of retrogressive ovary in class 2.   × 140.
FIG. 27. Transverse section of normal mature ovary.   × 22.5.
FIG. 28. Transverse section of retrogressive ovary in class 3.   × 205.
FIG. 29. Transverse section of oviduct of ovary in Fig. 28.   × 115.

Symbols :

bv	Blood vessel.
ctc	Connective tissue coat.
dfe	Disorganized follicular epithelium.
dno	Disintegrating ovum.
ee	External epithelium of gonad.
epc	Epithelial coat.
epo	Epithelium of ovarian cavity.
fed	Follicular epithelium of disintegrated ovum.
oc	Ovarian cavity.
mc	Muscular coat.
o	Ovum.
oo	Oöcyte.
ro	Remains of disintegrated ova.

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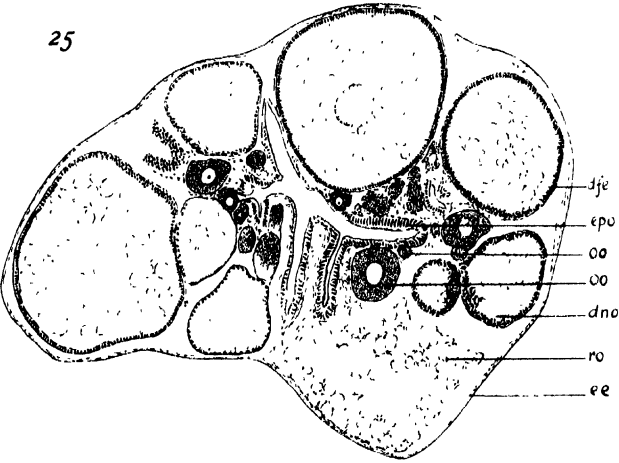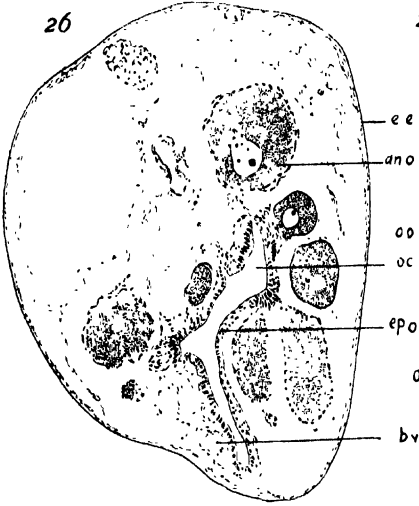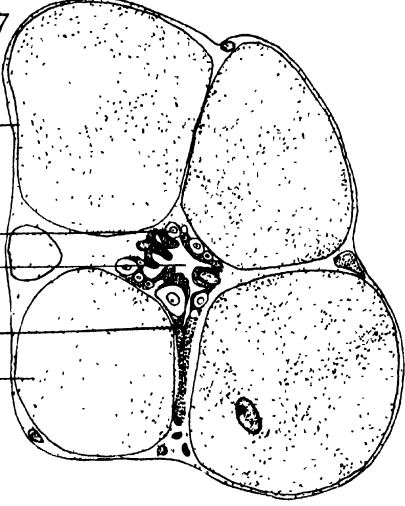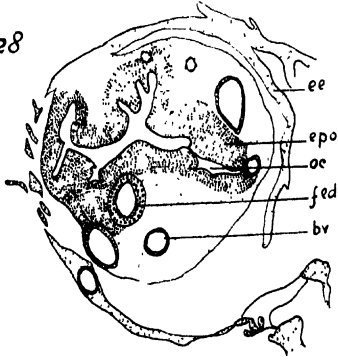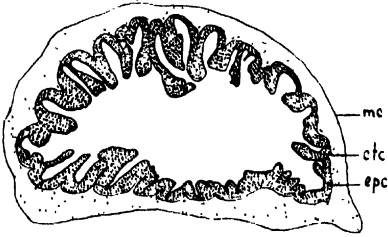
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mc
ctc
epc

Symbols:

ee	External epithelium of gonad.
m	Mesentery.
mch	Mesorchium.
nt	Nest of primordial germ cells.
p	Peritoneum.
pc	Peritoneal cells.
pgc	Primordial germ cells.
rc	Red blood cell.
sc	Sex cords.
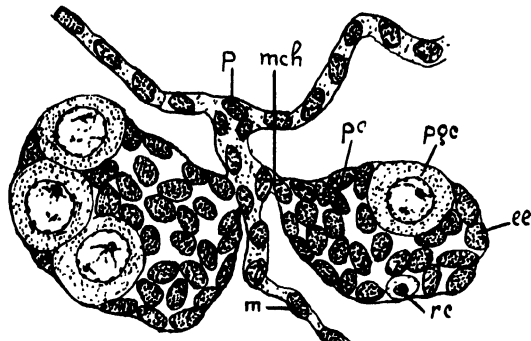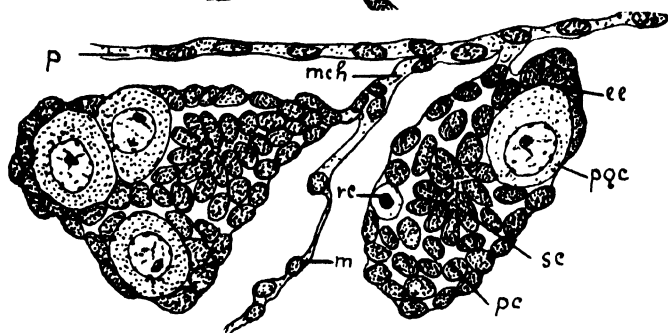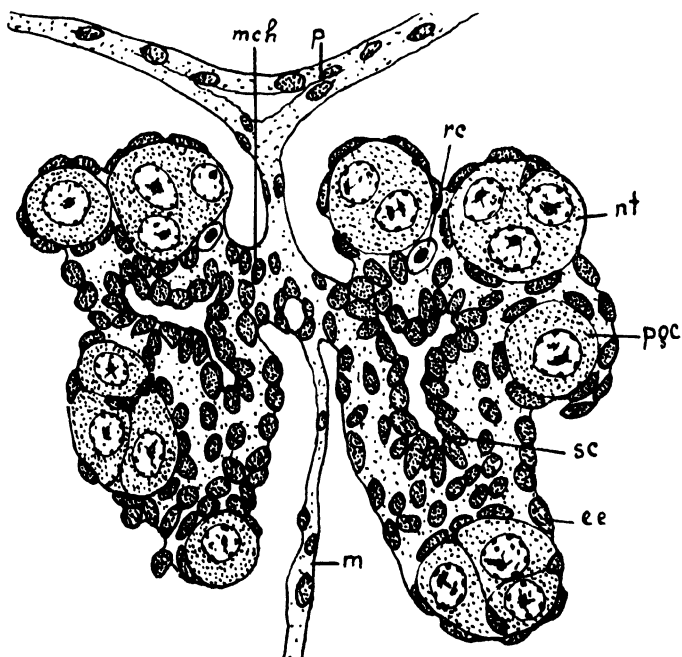
PLATE VI.

PLATE VII.

FIGS. 33–36. Transverse section of radial tubules to show transformation of tubule epithelium into definitive germ cells. × 1600.

Symbols:

et Epithelium of tubule.
fgc Fully formed germ cells.
mp Membrana propria.
lrt Lumen of radial tubule.
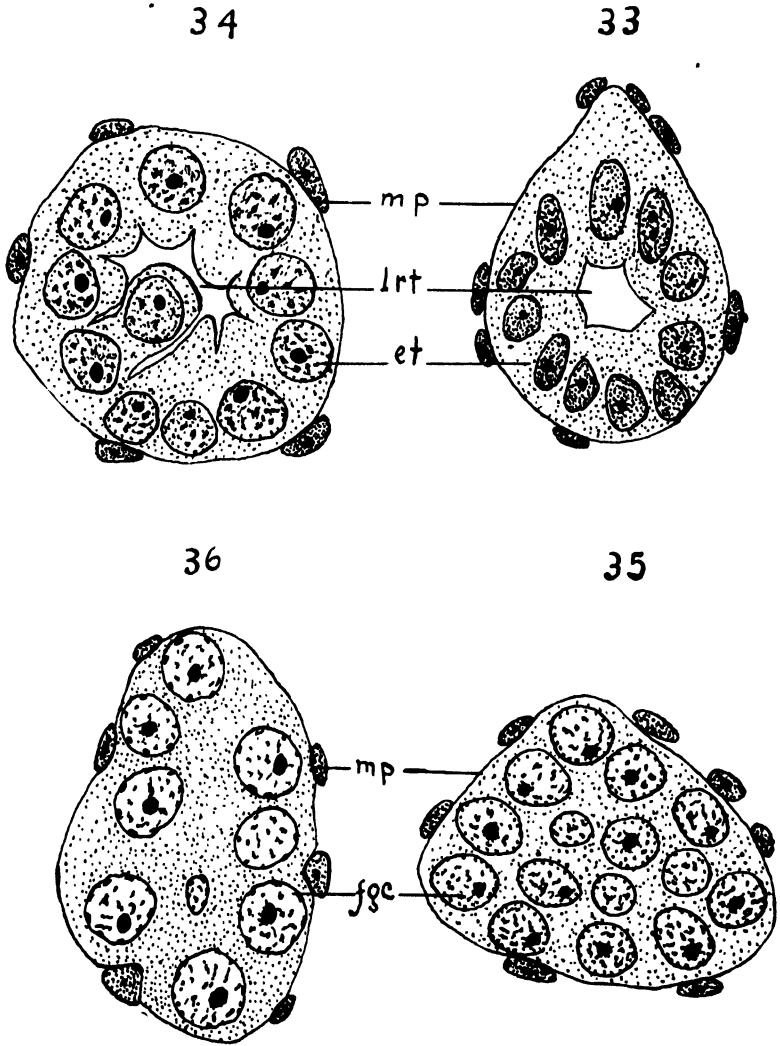
PLATE VIII.

FIG. 37. Portion of transverse section of adult testis showing spermato-
genesis.   × 410.

Symbols:

ppgc   Primordial germ cells on periphery of testis.
sds   Spermatids.
sph   Spermatophore.
spz   Spermatozoa.
ssc   Secondary spermatocytes.

PLATE VIII.

37

PPgc

SSC

sds

spz

sph

Symbols:

ac	Acinus or spermatocyst.
bsc	Branch of sex cord.
bv	Blood vessel.
d4r	Dorsal branch of fourth ray.
ee	External epithelium of gonad.
fep	Fin epithelium.
m	Mesentery.
mch	Mesorchium.
ntc	Nest of definitive germ cells.
p	Peritoneum.
ppgc	Primordial germ cells in periphery of testis.
sc	Sex cords.
sp	Spoon-like structures.
spd	Sperm duct.
t3r	Teeth of third ray.
t4r	Teeth of fourth ray.
v4r	Ventral branch of fourth ray.
1r–10r	First to tenth rays of anal fin.
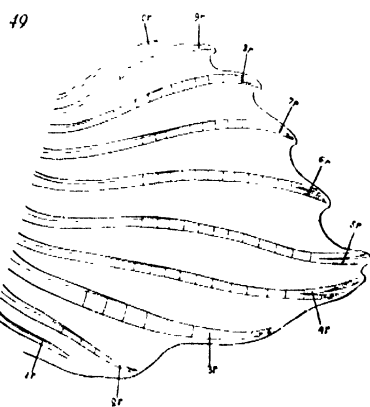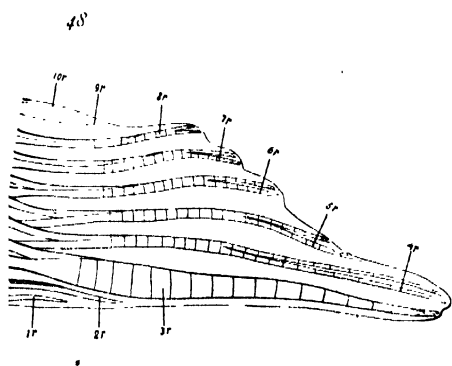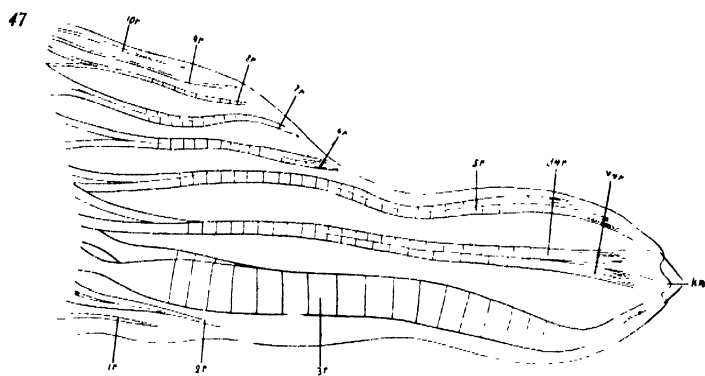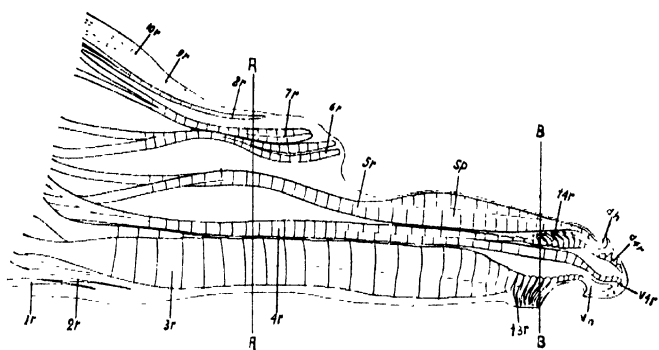
BIOLOGICAL BULLETIN

SELECTIVE COUPLING OF GAMMARIDS.

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ZOÖLOGICAL LABORATORY, RUTGERS COLLEGE.

The result of a method of breeding involving the formation of pairs according to some system of assortment, rather than upon a purely random basis, is readily seen to be of great importance, not only for the foundation of racial diversities, but also for the conservation of genetic stability (Romanes, 1906; Pearl, 1907[a]; Wright, 1921). Actual instances, however, of assortative mating, occurring under natural circumstances, have been little studied. And the matter is of interest beyond its strictly genetic bearings; for it is probable that through sexual coupling selective with respect to somatic features, there may in different species be achieved various automatic, adaptive, consequences non-genetic in character, but nevertheless significant racially (Crozier, 1918). In this respect selective pairing of protozoans and of metazoans may differ greatly as to their implications. The selective combination of gametes (*cf*. Jones, 1920) is a question quite distinct from that of assortment of individuals, and the two should not be confused.

Among metazoans relatively few cases of normal selective mating have been recognized, although Jennings (1920, p. 193) remarks that the propensity for like to mate with like is probably in some degree quite a general phenomenon. In *Paramecium* and allied ciliates, which have been most extensively investigated, there is a well-defined tendency toward conjugation between individuals resembling one another in size and in fission-rate, and to this extent at least structurally and physiologically akin (Pearl, 1907[a]; Jenning, 1911, 1920; Watters, 1912). This is in large part due to the fact that the mutual fitting of two individuals, requisite for conjugation, is mechanically possible only when these individuals

8   97

are closely similar. A like explanation holds for the selective pairing of the nudibranch *Chromodoris zebra* (Crozier, 1918, 1920) ; and perhaps also in the case of *Leptinotarsa,* although here Tower's (1906, pp. 238–243) account of the matter is not at all clear. Attempts to discover selective breeding involving other than size characters, among insects for example, have not been very successful. Kellogg (1906) describes observations upon 54 matings of *Hippodamia,* interpreted by him to signify that with respect to color pattern pairing is entirely at random. As Pearl (1907[b]) remarks in a review of Kellogg's note, the observations, on the contrary, actually do indicate possible assortment.

For preliminary study of pairing in forms possible to breed in the laboratory, we have examined naturally occurring couples randomly taken of two species of Gammarids: *Gammarus locusta* (Linn.) and *Dikerogammarus fasciatus.* (Say). Sixty-one pairs of *Gammarus* were obtained from one spot on the Staten Island shore of Raritan Bay; and seventy-one of *Dikerogammarus* were taken from the Raritan River at a point about a mile above New Brunswick.

It is possible to study size relations of members of the breeding pairs because the female is carried about by the male for a considerable time, fecundation occurring after an ecdysis by the fe-
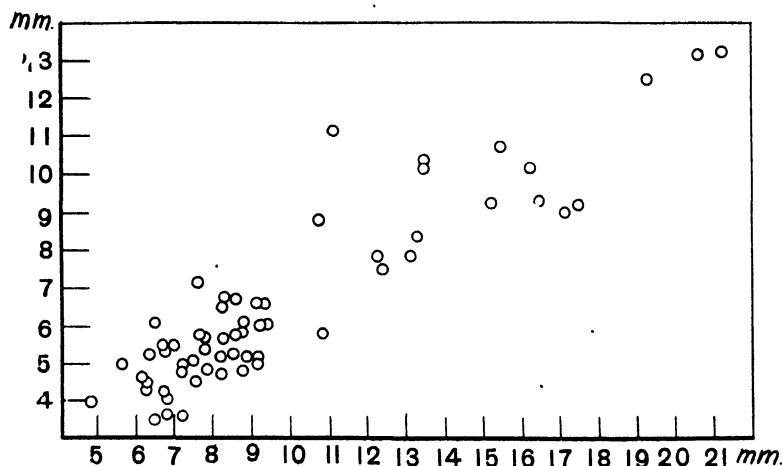
Fig. 1. Relation between the lengths of males and associated females in 62 pairs of *Gammarus locusta;* measurements in *mms.,* for each pair; ordinates, lengths of females; abscissas, lengths of males.

male. The length of each member of every pair secured was measured under low magnification, with the aid of an ocular micrometer; the total length, from anterior margin of cephalothorax to posterior margin of last abdominal segment, was measured along the curved dorsal outline.
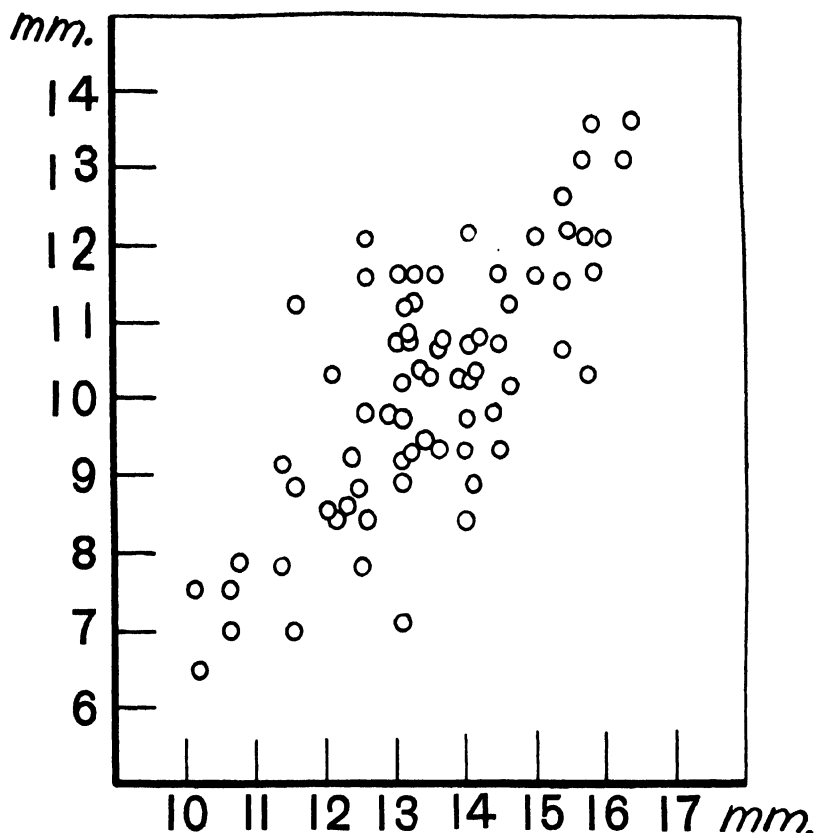
FIG. 2. Relation between length of male and of associated female in 71 pairs of *Dikerogammarus fasciatus;* lengths in *mms.,* for each pair; ordinates, lengths of females; abscissas, lengths of males.

The relation of the length of males to that of the associated females, in *Gammarus locusta,* is shown in Fig. 1 (Snyder and Crozier, 1922). Fig. 2 contains the corresponding observations for *Dikerogammarus fasciatus.* There is in each instance a high degree of selective coupling on the basis of length. For *G. locusta* the correlation index for size among members of mating pairs is

W. J. CROZIER AND L. H. SNYDER.
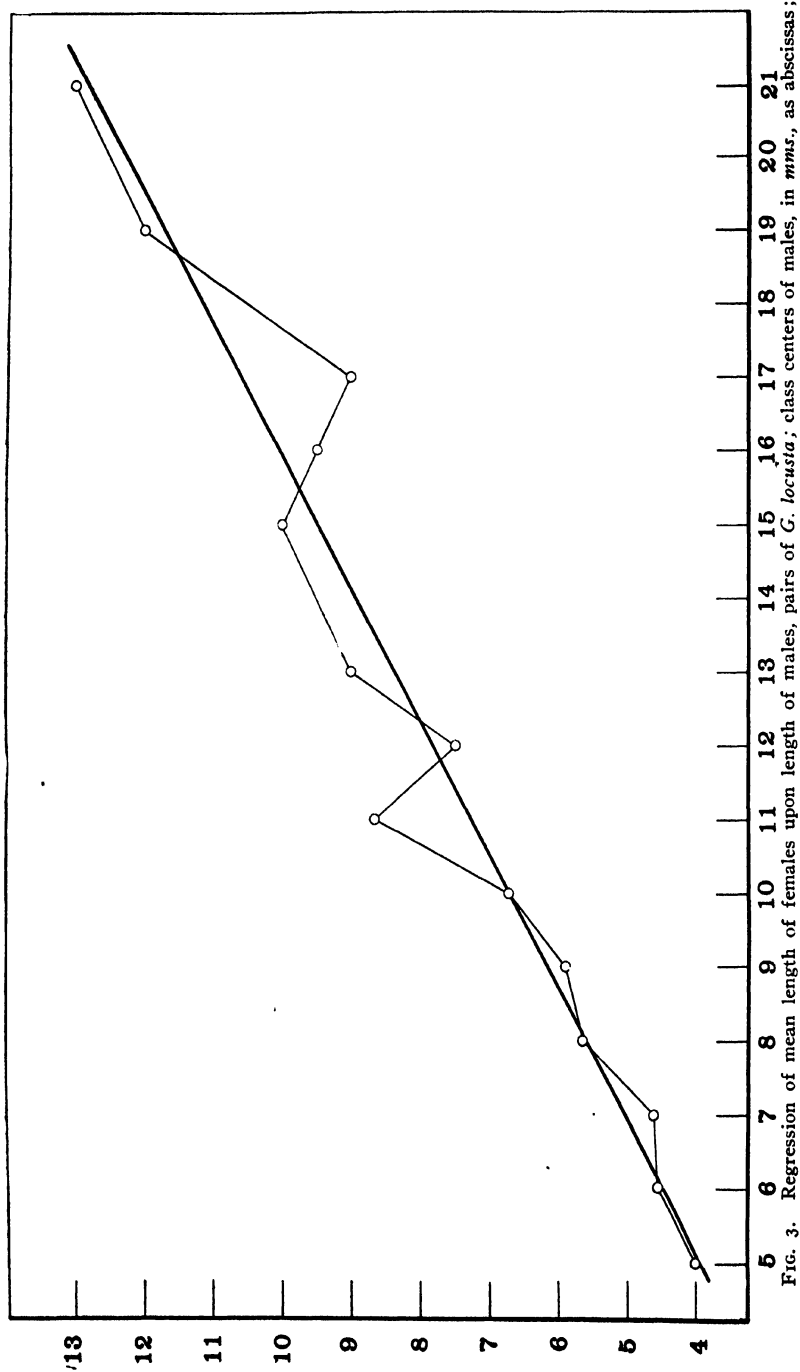
FIG. 3. Regression of mean length of females upon length of males, pairs of *G. locusta*; class centers of males, in *mms.*, as abscissas; ordinates, mean lengths of associated females.

$r = 0.914 \pm 0.014$; for *D. fasciatus,* $r = 0.690 \pm 0.042$.   Figs. 3 and 4 give the fitted lines of regression for mean lengths of the females associated with males of the corresponding length classes.

The formation of breeding pairs, according to Holmes (1903) and others who have studied the question of " sex-recognition " in gammarids and among other crustaceans,[1] is brought about in a purely mechanical way.   The initial encounter of male and female is by accident.   Males tend to clasp objects with which they come into contact.   A male seized by another male struggles until freed. A female, on the contrary, is passive when clasped, with abdomen and thoracic legs flexed, the whole body compact.   This account
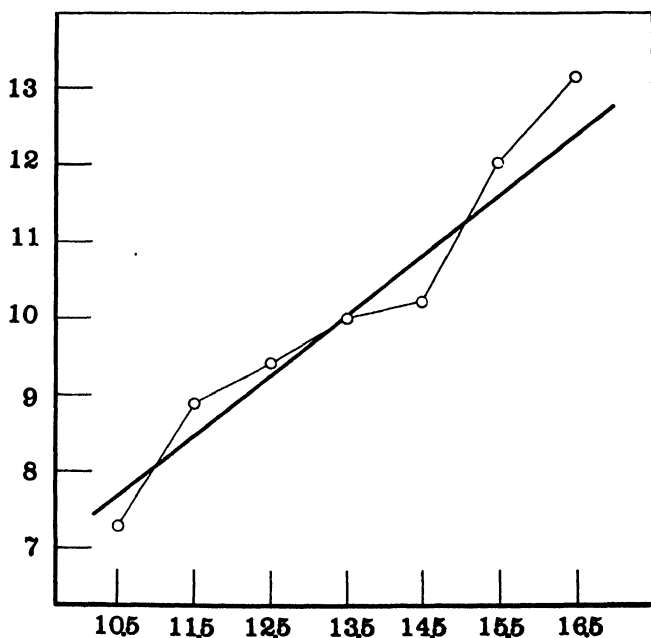
FIG. 4.   Regression of mean length of females upon length of males, pairs of *D. fasciatus,* lengths in *mms.,* class centers of males, abscissas; ordinates, mean lengths of associated females.

we can confirm for the species dealt with in our measurements. There is clearly a good mechanical reason for the formation of couples in which large males carry large females, and small males carry small females.   Holmes (1903) observed that a small male

[1] Holmes (1903, 1909); Pearse (1909); Andrews (1910); Chidester (1911).

gammarid had difficulty in carrying a large female, and could not clasp her successfully unless his appendages were able to reach around the body of the female. It is not so clear, at first sight, why small females are not found carried by large males. But closer inspection shows that the dactyls of the male pereiopods are neatly inserted under the edges of the coxal plates of the captive female. We incline, therefore, to the view that the graded correlation between the sizes of members of pairs is determined by mechanical features of the clasping process. The magnitude of the correlation indices found in these cases is in general agreement with those of the indices reported for *Paramecium* and for *Chromodoris*, in which also there is involved a purely structural adjustment.

The possible result of the selective coupling, in relation to number and size of offspring, remains to be studied. It is known that the number of eggs carried by a female gammarid varies directly with her size, hence it is not impossible that a phenomenon like that suggested in the case of *Chromodoris* (Crozier, 1918) may be involved here also. If the economical utilization of gametes be the chief consequence of selective coupling by sizes, we deal with an adaptive mechanism not necessarily involving factorial inheritance. The possibility of the latter complication can be tested experimentally. It is not out of place to call attention to another phenomenon in which selective pairing may well play an evolutionary rôle. The imaginal size of certain parasitic insects is known to be determined by the size and the rate of development of the particular species of host insect in which their larvæ grow and pupate (Keilin, 1915; Haviland, 1922). This is due to a developmental correlation between the pupation of the larval host and of its contained larval parasites. If adult insects, of the same species, differing in size through this means, practice selective mating and are by some agency compelled to oviposit in a host of the type from which they were themselves reared (*cf.* Wheeler, 1922), a basis is clearly afforded for the foundation of divergent types. This possibility may be examined experimentally; it is profitable, in the meanwhile, to study the conditions of assortative mating in a variety of types.

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THE EFFECT OF TEMPERATURE, FOOD, AND THE AGE OF THE CULTURE ON THE ENCYSTMENT OF *DIDINIUM NASUTUM*.

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INTRODUCTION.

Investigations on encysted organisms clearly indicate that length of life and lethal desiccation, temperature, and chemical concentration are greatly extended by encystment. Baker (1771) maintains that the rotifer, *Tylenchus,* revived after it had remained in a dried condition on the surface of grains of wheat for 27 years. Doyere (1842) found that rotifers kept for 17 days in a desiccator, followed by 28 days under the belljar of an air-pump with a pressure of only 5 to 6 cm. of mercury, and thereafter thoroughly dried in direct sunlight, and then subjected to a temperature of 140° C. were still viable. Mast says (1917): " Cysts of *Didinium* kept sealed air-tight in a 10 c.c. vial for nearly five years were still viable." Bodine found that the cysts of *Colpoda* withstand extraordinary concentrations of various acids and narcotics as well as remarkably high temperatures.

The results obtained in observations on the cause of encystment are, however, much less conclusive. Root maintains that the encystment of suctoria is due to lack of food. He says (1914): When left without food for several weeks, Podophryæ become smaller and finally encyst in situ." Mast obtained similar results in experiments on *Didinium,* but not invariably. He says (1917): " Encystment in *Didinia* can usually be induced at any time by cutting off the food supply. But it frequently occurs when there is an abundance of food present and sometimes it does not occur when there is none. This is especially true for the cultures in which conjugation has been prevented for a considerable number of generations." Miss Carter (1919) seems to think that abundant food is essential for encystment of *Amœbæ,* but that low tem-

perature is not, although she found encysted specimens during the winter months. Miss Hogue contends that the accumulation of waste products is the primary cause of encystment. She says, referring to *Amœba limax* (1914) : "The condition under which they encyst seems to point to a weakened vitality. It seems as though the digestive fluid has not been formed in sufficient quantity, owing to the rapid division. So it is not the lack of food, but rather the loss of power of assimilation." And (1917, p. 571) : "When the accumulation of waste product is very great, and the *Amœba* have multiplied so fast that there is no further place for them to go, they encyst. I have frequently observed a culture dish containing thousands of *Amœbæ*, with plenty of food, soon become covered with cysts, the *Amœbæ* having often encysted over night."

It is generally held that encystment is a protective adaptation. Thus Calkins says (1910, p. 40) : "This is a special adaptive process by which the organisms are enabled to survive when the environment is unsuitable." (P. 90) "There is a general agreement that its object is to protect the individual during periods of drought, cold, or periods of reproduction." (P. 192) "It occurs when the animal is in danger of drying, or in some cases before division, in others for the purpose of digesting a full meal." Minchin (1917) expresses similar views, as does also Jordan. Jordan says (1918, p. 73) : "It serves to tide the species over a period of dryness, famine, or unsuitable temperature, and to preserve alive in a hostile environment a sufficient number of individuals until such time as favorable conditions recur. The spore-stage is in fact physiologically analogous to the periods of hibernation and estivation among the higher forms of life. In this resting state the living matter of the spore may remain dormant for years or even for decades."

It seems evident, then, that many investigators hold that encystment is induced by adverse changes in the environment, and that it protects the organism against unfavorable conditions. The evidence presented in favor of the first of these contentions is, however, not strong. A thorough experimental investigation of the relation between the environment and encystment is, therefore, highly desirable.

In this paper we shall present the results obtained in experi-

ments on the effect of temperature, food, and the age of the food-culture on the encystment of *Didinium nasutum*.

MATERIALS AND METHODS.

The didinia used in these experiments were all derived from cultures started from a stem-culture kept continuously in the Laboratory. Battery-jars were used as containers for these cultures. From time to time, as the supply of food in the culture-jars became depleted, a portion of the liquid was replaced by a corresponding quantity of a fresh, vigorous, paramecium culture. In this way the didinia were kept continuously in a flourishing and active condition.

The effect of temperature and food on encystment was ascertained as follows: Into each of five square watch-glasses, previously sterilized, was placed 3 c.c. of culture fluid containing numerous paramecia, and into each of five similar watch-glasses 3 c.c. of culture fluid taken from the same jar, but filtered so as to remove all paramecia and other organisms that might serve as food for *Didinium*. To each of the watch-glasses thus prepared there were added vigorous didinia, usually five, all taken from the same jar. Two of the watch-glasses, one with and one without food, were then placed into each of five thermostats maintained at different temperatures as indicated in Table I. All of these cultures were examined for cysts once every day until they died out. In this experiment there were consequently under observation simultaneously ten didinia cultures which were precisely the same with the exception of food and temperature. This experiment was repeated a number of times as indicated in the accompanying tables.

In a number of additional experiments there were fewer cultures and temperatures under simultaneous observation. In an extended series of experiments made after the main part of the work was completed there were only two cultures, one with and one without food, both at 27° in each experiment. Cultures containing fluid taken from the jar which had contained the didinia used in the tests were added to some of the thermostats in a few experiments. This, however, had no appreciable effect on encystment.

The results obtained in all of these experiments are summarized in Table I. These results show conclusively that at 25°–30° en-

TABLE I.

THE EFFECT OF TEMPERATURE AND FOOD ON ENCYSTMENT IN DIDINIUM.
Note that the percentage of encystment is greater in the cultures with food
than in those without and that it is greatest at 25–30°, the optimum tempera-
ture for reproduction.

Temp.	With Food.			Without Food.			Ratio of Percentages of Encyst-ment.
	No. of Cultures.	No. of Cultures Encysted.	Percentage of Cultures Encysted.	No. of Cultures.	No. of Cultures Encysted.	Percentage of Cultures Encysted.	
4–16°....	14	0	0	11	0	0	
20–23°....	26	14	53.8	22	4	18.18	3.23
25–30°....	18	15	83.3	8	3	37.5	2.22
27°........	51	42	82.3	57	22	38.6	2.13
30–35°....	25	17	68.0	26	7	26.92	2.52
39°....	16	6	37.5	9	1	11.1	3.38

cystment occurred in a much greater proportion of the cultures
than at any other temperature, both in those with and in those
without food, that at all of the temperatures excepting the lowest
it occurred in a much greater proportion of the cultures which
contained food than in those which did not, and that the difference
in the extent of encystment in the cultures with and without food
was least at 25°–30°. They show that at 39°, which is only a few
degrees below the maximum, there was but little encystment, and
that at 4°–16° there was none at all.

In the preceding experiments the didinia died out in relatively
more cultures at the higher and the lower temperatures than at the
others. At 4°–16° and at 39° they died out in over half of the
cultures in two days, while at 27° they died out in less than one
tenth of the cultures in the same time. Moreover, the death rate
was greater at all of the temperatures in the cultures without food
than in those with food. In two days at 39° two thirds of the
cultures without food died out and only one half of those with
food, at 30°–35° a little over one half of those without food and
less than one eighth of those with food, at 27° nearly one sixth of
those without food and less than one twenty-fifth of those with
food, etc. Furthermore, reproduction took place more rapidly at
25°–30° than at the other temperatures. At 25°–30° the fission
rate ran up in some instances to 8 a day. At 39° it never exceeded
one or two a day, and at the lower temperatures of 4°–16° there
was no reproduction at all.

In all of these experiments the didinia were rather suddenly sub-jected to the different temperatures.   It was thought that the lack of encystment at the extreme temperatures might have been due to the sudden change.   An extended series of tests was consequently made in which the temperature of some of the cultures was very gradually reduced to 4°–16°, and that of others very gradually raised to 39°.   The results obtained in all of these tests were, how-ever, essentially the same as those obtained in the earlier experi-ments.

All of the results obtained consequently indicate that encystment in *Didinium* takes place most readily under conditions of tempera-ture and food which appear to be optimum for reproduction.   The results are, however, not conclusive in reference to the question of the effect of food.   An abundance of food was added in all of the experiments when they were set up and in some more was added later.   In some cultures there was still an abundance of food pres-ent when encystment occurred, but in others there was none.   The number of each was unfortunately not recorded.   It is conse-quently evident that in some of the cultures which contained food encystment may have been due to absence of food.   In the cultures with food there was a much greater increase in the number of didinia than in those without food.   Rapid and extensive repro-duction of didinia confined to a small space seems to favor encyst-ment, and it may be that this is owing to accumulation of waste products.

In attempting to ascertain the effect of the age of the food-cul-ture on encystment experiments were carried out as follows: Tim-othy hay was added to tap-water, spring-water, and distilled water in three large flasks, one gram to 100 c.c.   These flasks were then kept for 30 minutes at the boiling point.   After they had cooled to room temperature, usually the following day, a large number of paramecia in a small amount of liquid was added to each flask and the contents poured into battery-jars.   These jars were then placed side by side and kept at room temperature.   Three more cultures were prepared precisely the same way two days later and also four days later; so that at this time there were at hand three sets of paramecia cultures, one just completed, one two days old, and one four days old.   A given amount of solution containing many para-

mecia was now taken from each of the nine cultures and put into nine square watch-glasses, each containing five vigorous didinia, all taken from the same jar. These watch-glasses were put into a thermostat kept at 27°–28°. The number of cultures in which cysts occurred was recorded daily, as well as the number of cysts in each and the condition of the paramecia.

Other sets of watch-glass cultures were prepared and treated precisely like this on the following days. A summary of the results obtained in all of these are presented in Table II.

TABLE II.

THE RELATION BETWEEN THE AGE OF THE FOOD-CULTURE AND ENCYSTMENT
IN DIDINIUM.

Age of Food Culture in Days.	No. of Didinia Cultures.	No. of Cultures Encysted.	Average Time Required for Encystment in Days.	Percentage of Cultures Encysted.
0	11	2	3	18.1
1	8	5	3-2/5	62.5
2	11	4	3	36.3
3	14	7	3	50
4	14	10	3-1/5	71.4
5	20	15	3-1/5	75
6	15	14	3-1/14	93.3
7	21	11	2-6/11	52.3
8	18	12	3-1/3	66.6
•9	13	9	2-2/9	69.2
10	15	7	3	46.6
11	10	4	3	40
12	10	4	2-3/4	40

By referring to this table it will be seen that as the culture medium increased in age the percentage of the number of cultures in which encystment occurred increased to a maximum, after which it decreased. It is well known that the chemical composition of protozoa cultures changes with age. It is consequently evident that the increase and decrease in the percentage of encystment noted must have been due either to this change or to a change in the quantity or the quality of the food. The amount of food was, however, practically the same in all of the didinia cultures, but the quality may have been different. It is therefore impossible to say whether the change in the percentage of encystment was due to a change in the chemical composition of the culture medium or to a

change in the quality of the food. However this may be, encystment occurs freely in the culture media which are very favorable for growth and reproduction of paramecia. The maximum was found in cultures six days old, when the paramecia were very abundant and vigorous. Whether or not fission rate was at a maximum at this time was, however, not ascertained.

SUMMARY.

1. Didinia encyst most readily at a temperature of $25°-30°$, which is also the optimum temperature for growth and fission.

2. They do not encyst in temperature so low or so high that it is injurious. They do not encyst at all below $16°$ and rarely above $39°$.

3. They encyst more freely in cultures supplied with food than in those without food, but this is probably due to greater increase in numbers, resulting in greater accumulation of waste material in the one than in the other.

4. They encyst most readily in culture media, which are probably most favorable for growth and reproduction of the paramecia on which they feed.

5. Encystment serves as protection against unfavorable conditions in reference to food and temperature, but such conditions do not facilitate encystment.

6. Encystment is probably induced by the accumulation of excretory waste material.

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FEEDING REACTIONS IN THE CILIATE, *DILEPTUS GIGAS,* WITH SPECIAL REFERENCE TO THE FUNCTION OF TRICHOCYSTS.

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Contents.

1. INTRODUCTION.

Food-getting is the first necessity of the living thing, and the chief end toward which the fundamental structures of the body are directed, and this, whether in the highest mammal or lowest protozoan, becomes the chief economic problem to be solved. " Food-getting, therefore, more than any other function of the body, has been the most influential in leading to morphological development." Thus wrote one of the leading investigators in the field of protozoölogy (Calkins, '10).

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The feeding reactions of animals have long been a favorite study for students of animal behavior. More recently the reactions of certain protozoa have been intensively studied with the hope that such problems as the choice of food would be reduced to their simplest terms in these unicellular animals which in other respects apparently stand near the bottom of the scale of development.

Ehrenberg ('38) was undoubtedly one of the first to make observations on the ability of protozoa to choose their food. He records experiments on the ingestion of carmine by various organisms in an attempt to show that protozoa do not select their food. Entz ('88) and others of his time were, however, strong in their contention that infusoria are able to select their food, ingesting certain kinds and rejecting others in a systematic way. Bütschli ('89) supports Ehrenberg's view. He concludes that protozoa do not possess the power of choice, and Verworn ('89) likewise concludes, on the basis of rather extensive experiments, that there is no selection.

Jennings ('02) states that *Vorticella* and *Stentor* probably do not have the power of selecting their food in any precise way. Mast ('09) in his work on *Didinium* shows that the apparent choice of food on the part of this organism is due to the fact that the seizing organ will adhere to the surface of some organisms and not to others. The didinia come in contact with all sorts of objects in their random swimming and " select " as food only those to which the seizing organ will adhere.

Schaeffer ('10) in his work on *Stentor cœruleus* concludes that this organism exercises a very definite selective power and discriminates very accurately between organisms and indigestible particles, and that it discriminates even between different organisms. He contends that it selects its food on a tactual basis, and apparently not on a chemical one. The same worker ('16) reports some experiments on *Amœba* and maintains (p. 562) that although *Amœba* eats insoluble substances, there is a slow process of learning in favor of selection. He ascribes to the endoplasm of *Amœba* a more specific power of discrimination than to the ectoplasm, and also maintains that movement of an object is a very important factor in determining whether or not it shall be eaten.

Calkins ('10) says, " while most of the protozoa wait until the

prey comes to them, and take what they can get, others are pre-datory and go in search of food. These are the most interesting of all protozoa, for they are occasionally too fastidious apparently to take the ordinary run of microscopic wilds, but seem to select their food with all the care of a gourmand." As an example of this type he describes the reactions of *Actinobolus radians*.

Miss Moody ('12) in her study of *Actinobolus* and *Spathidium* asserts that they "subsist exclusively on a special type of ciliate. *Actinobolus* awaits the coming of *Halteria grandinella* before mak-ing use of its weapons of offense," while *Spathidium* swims about "with seeming indifference to all food material except the little ciliate, *Colpidium colpoda*." She concludes that "the protoplasm of these organisms has become modified chemically and physiologi-cally to such an extent that a reaction to one kind of protoplasm only is possible; in other words, forms like *Actinobolus* and *Spathidium* have become "educated through 'error' to the selection of one species of food each, namely. *Halteria grandinella* and *Colpidium colpoda*."

Metalnikow ('12) contends.that if paramecia are fed for some time on a non-digestible substance, they take in gradually less and less, until finally they refuse it entirely under all conditions, but that they nevertheless take in other substances just as before. He shows that in the case of feeding on carmine this power of selection is lost at the time of division. He also shows that there is a de-cided power of discrimination between substances already within the body; for some substances are quickly excreted, while others remain within the body for a considerable length of time.

In his studies of one of the Suctoria, *Podophrya collini*, Root ('14) maintains that there are several definite factors which deter-mine the selection of food in this organism. He shows that the character of the outer surface of certain organisms as to physical and chemical constitution, mucus secretion, etc., prevents the attachment of the seizing apparatus. He shows, moreover, that the size, the activity, and the characteristic behavior of certain organisms in relation to the sessile habits of *Podophrya collini* are also determining factors.

From this brief review of some of the more general literature in this field it is evident that selection of food has thus far been

positively demonstrated in only a very few forms, while in general
it would appear that most workers have supported the opposing
view.

There are only a few incidental references in the literature to
the feeding habits of *Dileptus gigas*. Bütschli ('89) says its food
is " sehr grob," and is quoted by Calkins as saying that it feeds
on ciliates alone. According to Wrzesniowski ('70), "*Dileptus
gigas* is a voracious animal which feeds only on living food, preying
especially on *Stylonychia.*" Pritchard ('61) says it feeds largely
on green monads, because of which it is often of a green color.
Hausman ('17) says "*Dileptus* is surely the king of beasts among
the ciliated protozoa. It is entirely carnivorous and its appetite is
apparently insatiable. The prey is stung by well-developed tricho-
cysts, and if too large to be swept into the buccal cavity by the cilia,
it is forced in by the writhings of the neck " (proboscis).

It is clearly evident that there are a number of different views
concerning the feeding habits of this infusorian, all of which are
apparently based on purely incidental observations. Does *Dileptus*
feed on ciliates alone, or even on living food only, which would
involve the power of choice of food? Does it paralyze its prey
by means of trichocysts? What is the nature of these structures?
These are the problems which are considered in the observations
and experiments which comprise the material presented in this
paper.

The work was begun at the suggestion of Professor S. O. Mast,
to whom I am deeply indebted for many helpful suggestions con-
cerning the experiments made and for much valuable criticism dur-
ing the preparation of this paper.

2. MATERIAL AND METHODS.

Dileptus gigas is one of the holotrichous ciliates belonging to the
family Tracheliidæ. It is one of the largest of the more common
protozoa, often measuring over 600 micra in length. It possesses
an elongated body, sharply pointed at the posterior end, and at the
anterior end drawn out into a long proboscis which is frequently
as long as the body itself. The mouth opening is located at the
basal end of this proboscis, and has a circular aperture with a short
funnel-shaped gullet leading from it (Fig. 1). Both these struc-

tures are capable of enormous expansion at the time of feeding. Normally, however, they are closed except for a pit-like cavity which is always present. A cytopyge is sometimes discernible near
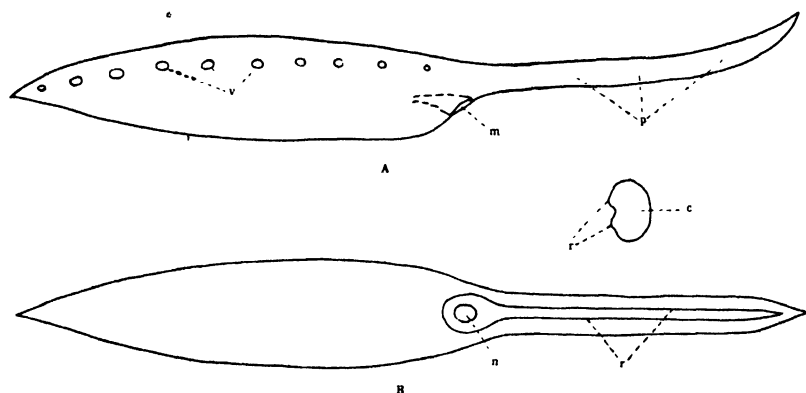
FIG. I. Diagrammatic sketches of *Dileptus gigas:* *A*, side view; *B*, oral view; *v*, contractile vacuoles; *p*, proboscis; *m*, mouth; *c*, cross section of proboscis; *r*, bands of large cilia.

the posterior end, at which place fæcal material often collects in a large vacuole from which it is sporadically discharged. There are numerous contractile vacuoles of which the larger ones are arranged in a series near the aboral surface. Thin contractile fibrillæ extend around the body in the form of a flat spiral, effecting about one complete turn for the entire length of the body. The entire body is covered with short cilia which run in rows parallel to the fibrillæ. On the ventral surface of the proboscis, which is somewhat flattened, the cilia are considerably thicker and longer than elsewhere, especially along the edges, where they are in the form of two bands. These extend backward in such a way as to meet and form an arch just behind the mouth opening. Since all these cilia beat backward under normal conditions, a decided current is produced in the groove between these two bands of large cilia. This current starts at the tip of the proboscis and runs back to the mouth, where it ends in a sort of vortex, due to the action of the band of cilia which partially surrounds this pit-like orifice as above described. Structures which have been quite generally described as trichocysts are found in the oral surface of the proboscis. They can be seen only indistinctly in living mate-

rial. There are at least several hundred of these structures, all of which are arranged in a band extending from the mouth to the tip of the proboscis, approximately in the mid-line of its oral surface.

There has been much discussion concerning the nuclear condition of this organism, but recent investigators appear to be agreed that *Dileptus* possesses a distributed nucleus. An account of this condition will be omitted here, as it is planned to present a discussion of the nuclear phenomena during the life history of this organism in a separate paper.

Dileptus is never at rest. It is always swimming, as a rule quite slowly unless disturbed. In cultures it is observed to spend most of its time swimming slowly just above the debris at the bottom of the dishes. Apparently a like condition obtains in nature, for if *Dileptus* is found in a pool, samples taken from near the bottom' contain many more specimens than those taken at higher levels. It is observed to progress with its posterior end close to or in contact with the bottom, while its anterior end is always at a noticeable elevation. It proceeds with its proboscis ahead, continually waving it now this way, now that, as if in search of food. It also rotates slowly on its longitudinal axis. This rotation, in connection with the searching movements of the proboscis, enables it to explore a very large area.

In January, 1919, a few specimens of *Dileptus gigas* appeared in an old " paramecium-culture " in which active fermentation had long since ceased. It can not live long in any culture in which active fermentation is taking place, nor can it thrive in cultures rich in organic food supply. Consequently, very dilute infusions were made, and these were usually inoculated with *Euglena gracilis* before introducing *Dileptus*. In this manner the original culture was kept for more than fifteen months.

In nature I have found them in the large pond on the Homewood Campus of the Johns Hopkins University, Baltimore, and also in the " school-house " pond near the Biological Laboratory at Cold Spring Harbor, L. I. They were found also in the Cedar Swamp pond at Woods Hole and in the outlet channel of Creve Cœur Lake near St. Louis, Mo. In all these ponds at the time *Dileptus* was found there was but little organic decay taking place and the water was " relatively pure."

In preparation for the experiments described in the following pages, a number of the organisms, usually about fifteen, were transferred with a capillary pipette from the stock-culture to small dishes containing about 5 c.c. of spring water. They were left in this water without food for from 2 to 3 days. During this starvation process about ten per cent. usually encysted and the rest diminished considerably in size, became very hungry, and were consequently in excellent condition for observation on the selection of food and the process of feeding. When kept longer, without food, they continued to diminish in size until in the course of a week they were reduced to less than one tenth of their former length and probably to less than one one-hundredth of their former volume, after this they disintegrated unless this was prevented by opportune feeding.

All the experiments on the selection of food were conducted as follows: A designated number of starved dilepti, usually fifteen, were placed in a small watch-glass with about 3 c.c. of spring water. A drop of a concentrated suspension of the substance to be tested was then added and the whole thoroughly mixed. The feeding reactions of some few of the specimens were carefully observed for longer or shorter periods of time. At the end of twenty minutes all the dilepti were taken out of this medium by means of a small pipette and introduced into a like volume of spring water. They were then observed, either individually or two at a time, on a slide under a magnification of about 300 diameters, and the number of vacuoles in each was carefully counted. In this way various living organisms and numerous inanimate particles were tested.

The protoplasm of *Dileptus* is quite opaque under ordinary conditions of culture. Occasionally a culture was obtained in which the organisms were relatively transparent and it was only with such material that the feeding experiments were made, for even under optimum conditions there was some difficulty in seeing precisely what passed into the body unless it was specifically colored as, *e.g.*, carmine or india ink. Another factor which favored observation on the amount of food ingested was the characteristic large size of the first vacuoles formed, whenever an organism was introduced into a new medium, especially after a period of starvation. Such large vacuoles are very specific.

3. EXPERIMENTS ON SELECTION OF FOOD.

A.   Inanimate Substances.

In experiments on inanimate substances it was desired to use only insoluble and non-toxic materials.   The following substances were tested: carmine, chalk, sand, powdered glass, and india ink. Three of these, namely, carmine, glass, and india ink, appeared to be more favorable than the others, because of the fact that vacuoles filled with these substances were readily distinguishable.   The results obtained in ten experiments with each of these three substances are given in Tables I., II., and III.

Table I. contains the results obtained with carmine.   This table

TABLE I.

EXPERIMENTS ON CARMINE.

Table showing results obtained in feeding *Dileptus* on carmine.   In the five columns under the headings 0–4, are indicated the number of individuals which formed 0, 1, 2, 3, 4, or more vacuoles containing carmine, respectively, during the twenty minutes of the experiment.   Carmine was ingested by 97.5 per cent. of the individuals but in only 17 per cent. was there more than one vacuole containing carmine formed.

Experiment Number.	Total Number of Dilepti.	Number of Carmine Vacuoles.				
		0	1	2	3	4 or More.
1..........	15	0	14	1	0	0
2..	15	0	12	2	1	0
3	15	0	8	7	0	0
4	14	0	11	3	0	0
5	15	1	13	1	0	0
6..	15	1	12	2	0	0
7...... .	13	0	12	1	0	0
8.........	15	1	11	2	1	0
9	16	0	13	3	0	0
10..........	16	0	14	1	1	0
Average.....	14.9	.3	12	2.3	.3	0
Per cent.....		2	50.5	15	2	–

shows that only three of the one hundred and forty-nine individuals tested did not ingest carmine, that more than eighty per cent. of the total number tested formed one vacuole, while fifteen per cent. formed two vacuoles each.   Thus it is evident that carmine is eaten, but only in small quantities, for in only seventeen

per cent. of the individuals tested was there more than one vacuole containing carmine formed.

Table II. contains the results of ten experiments with powdered

TABLE II.

EXPERIMENTS ON BLUE GLASS

Table showing the results obtained in feeding *Dileptus* on powdered blue glass. In the five columns under the headings 0–4, are indicated the number of individuals which formed 0, 1, 2, 3, 4, or more vacuoles containing blue glass, respectively, during the twenty minutes of the experiment. Glass was ingested by 89.3 per cent. but in only 12 per cent. was there more than a single vacuole containing glass formed.

Experiment Number.	Total Number of Dilepti.	Number of Vacuoles Containing Glass.				
		0	1	2	3	4 or More.
1..........	15	4	8	2	1	0
2..........	14	3	10	1	0	0
3..........	15	0	10	3	1	1
4........ ..	15	0	15	0	0	0
5...	15	1	14	0	0	0
6	15	3	9	2	0	1
7...	16	0	16	0	0	0
8...... . ..	15	0	14	1	0	0
9........ .	15	4	11	0	0	0
10...... . .	15	1	9	3	1	1
Average.....	15	1.6	11.6	1.2	.3	.3
Per cent.....		10.7	77.3	8	2	2

blue glass. It shows that eighty-nine per cent. were observed with vacuoles containing glass; that although so large a percentage had ingested glass to some degree, yet only twelve per cent. formed more than one vacuole; and that only two per cent. were observed with more than three vacuoles containing glass. It is therefore evident that while glass is ingested, it is taken only in very small amounts.

Table III. contains the results obtained with india ink. It shows that almost ninety-five per cent. contained at least one vacuole with ink particles in it, and that only twenty-seven per cent. had formed a second vacuole during the entire twenty minutes. We can conclude, therefore, that ink is eaten in small amounts by the great majority of specimens of *Dileptus,* but that only relatively few form more than two vacuoles containing this substance.

TABLE III.

EXPERIMENTS ON INDIA INK.

Table showing results obtained in feeding *Dileptus* on India ink. In the columns under the headings of 0–4, are indicated the number of individuals which formed 0, 1, 2, 3, 4, or more vacuoles each, containing India ink, during the twenty minutes of the experiment. Ink was ingested by more than 94 per cent., but less than 28 per cent. formed more than a single vacuole containing ink.

Experiment Number.	Total Number of Dilepti.	Number of Vacuoles Filled with Ink.				
		0	1	2	3	4 or More.
1..........	15	1	11	2	0	1
2..........	15	0	12	2	1	0
3..........	15	0	11	1	2	1
4	16	1	13	2	0	0
5	17	2	11	3	1	0
6..........	15	3	12	0	0	0
7	15	0	8	4	1	2
8..	12	1	7	3	0	1
9	15	0	3	5	4	3
10	15	0	13	2	0	0
Average. ...	15	.8	10.1	2.4	.9	.8
Per cent.....		5.3	67.3	16	6	5.3

In the experiments on all the other inanimate substances mentioned earlier, results were obtained which are, in the main, in harmony with those presented in Tables I., II., and III. All these substances, with the possible exception of sand, were ingested by a great majority of the individuals used in the tests. Experimentation with sand was very difficult owing to the fact that it settles very quickly, and that it is also difficult to see. My notes on the few experiments made record only thirty-two per cent. as having fed on this substance. The results obtained in experiments with chalk are almost identical with those obtained with glass. In the experiments on starch there does not appear to have been as sharp a decline between the number forming only one vacuole and those forming three or four vacuoles each within·the twenty minutes. In other words, the power of discrimination does not seem to be as well developed in regard to this substance as it is in regard to the others. These experiments on inanimate substances thus show quite clearly that *Dileptus* when hungry will ingest insoluble substances, but that usually only one vacuole is formed.

B.   Animate Substances.

In comparison with the results obtained in the above-described experiments on inanimate substances, those obtained in experiments on living material stand out in sharp contrast.   Experiments were

TABLE IV.

EXPERIMENTS ON *Euglena*.   ·

Table showing results of ten experiments obtained in feeding *Dileptus* on *Euglena*.   In the five columns under the headings 0–4, are indicated the number of individuals which formed, in twenty minutes, 0, 1, 2, 3, 4, or more vacuoles containing *Euglena*.   *Euglena* was ingested by more than 94 per cent. and a second vacuole containing this flagellate was formed by 89.3 per cent. of the individuals tested.

Experiment Number.	Total Number of Dilepti.	Number of Vacuoles Containing *Euglena*.				
		0	1	2	3	4 or More.
1..........	15	1	0	1	4	9
2..........	15	0	0	1	3	11
3..........	14	0	0	1	5	8
4.... .. .	12	1	2	0	4	5
5....	16	0	1	2	2	11
6	17	0	1	3	2	11
7.	15	0	0	1	5	9
8 . .	15	0	0	0	3	12
9 . .	16	6	3	2	3	2
10 ..	15	0	1	3	2	9
Average ...	15	.8	.8	1.4	3.3	8.7
Per cent. ...		5.3	5.3	19.3	22	58

made with most of the forms listed in Table VII.   The results obtained with all of these forms are essentially the same.   Those obtained with *Euglena, Colpidium,* and *Chilomonas* are presented in Tables IV., V., and VI.   By referring to these tables it will be seen that *Euglena* was ingested by 95 per cent. of the dilepti tested, 89 per cent. forming three. or more vacuoles, and 50 per cent. four or more; that *Chilomonas* was ingested by 98 per cent. of the dilepti tested, 70 per cent. forming three or more vacuoles; and that *Colpidium* was ingested by 99 per cent. of the dilepti tested, 84 per cent. forming more than one vacuole.   It is consequently evident that these organisms are ingested in large numbers by the majority of the dilepti tested.

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TABLE V.

EXPERIMENTS ON *Colpidium.*

Table showing results obtained in feeding *Dileptus* on *Colpidium.* In the five columns under the headings 0–4, are indicated the number of individuals which formed, in twenty minutes 0, 1, 2, 3, 4, or more vacuoles containing *Colpidium.* *Colpidium* was ingested by 99 per cent. and 84 per cent. formed more than one vacuole.

Experiment Number.	Total Number of Dilepti.	Number of Vacuoles Containing *Colpidium.*				
		0	1	2	3	4 or More.
1	14	0	3	5	6	0
2	15	0	2	4	3	6
3	12	0	3	2	5	2
4	11	0	0	4	6	1
5	15	1	5	3	4	2
6	15	0	1	7	2	5
7..	12	0	3	4	4	1
8.	12	0	2	3	6	1
9	15	0	0	4	8	3
10..	14	0	1	3	7	3
Average...	13.5	.1	2	3.9	5.1	2.4
Per cent.....		.7	14.8	28.9	37.7	17.7

TABLE VI.

EXPERIMENTS ON *Chilomonas.*

Table showing results obtained in feeding *Dileptus* on *Chilomonas.* In the five columns under the headings 0–4, are indicated the number of individuals which formed 0, 1, 2, 3, 4, or more vacuoles containing *Chilomonas* in twenty minutes. *Chilomonas* was ingested by 98 per cent. of the dilepti tested and 93 per cent. formed more than one vacuole.

Experiment Number.	Total Number of Dilepti.	Number of Vacuoles Containing *Chilomonas.*				
		0	1	2	3	4 or More.
1	14	0	1	3	4	6
2.	15	0	0	6	4	5
3....	15	0	1	3	4	7
4...	15	0	1	4	2	8
5	15	0	0	2	7	6
6..........	15	1	1	5	3	5
7.........	16	1	0	3	4	8
8..........	15	0	2	3	7	3
9	15	0	2	1	4	8
10..........	15	0	0	4	3	8
Average.....	15	.2	.8	3.4	4.2	6.4
Per cent.....		1.3	5.3	22.7	28	42.7

By comparing these results with those on inanimate substances as shown in Tables I., II., and III. it will be noted at once that the living material was ingested by a much greater percentage of the dilepti tested, and also that the average number of vacuoles formed during the twenty minutes of the experiment was almost three times as great in the experiments on living material as in those on inanimate substances.   It will also be noted that when feeding on inanimate substances *Dileptus* tends to stop feeding after having formed one vacuole, but that when it is feeding on animate substances it does not.   This clearly indicates some power of selection, for *Dileptus* refuses to take in useless materials while it ingests nutritive substances in large amounts.

Having thus observed that *Dileptus* can select between animate and inanimate substances, the question naturally arose as to whether there is any choice between different kinds of organisms. ˙ Many species of organisms were used in attempting to answer this question.   The same methods were used as described above, but only the results obtained in observations on the actual process of feeding were recorded.

The results obtained in these observation, in so far as they pertain to the problem of selection, are briefly summarized in Table VII.   By referring to this table it will be seen that *Dileptus* does

TABLE VII.

DISCRIMINATION BETWEEN DIFFERENT ORGANISMS.

Table giving results of feeding tests with Dileptus showing selection among living organisms.

I. Organisms Readily Captured.	II. Organisms Captured only Rarely.	III. Organisms Never Captured or Injured in Any Way
Euglena gracilis(?)	*Paramecium aurelia*	*Paramecium caudatum*
Trachelmonas	*Frontonia*[1]	*Frontonia*[1]
Amœba	Rotifers	*Euplotes*
Halteria	*Stylonychia*	*Nassula*
Urocentrum turbo	*Spirostomum*	
Chilomonas paramecium		
Colpidium		
Colpoda		
Stentor cœruleus		
Stentor polymorphus		

[1] Two races of *Frontonia* were used, the one being entirely immune to the attacks of *Dileptus*, and the other only partially.

not. capture all organisms, but selects from among the different kinds of living organisms in accordance with the grouping shown. The basis for this grouping will be more readily understood after presenting a few observations on the mechanics of feeding and the function of the trichocysts.

4. OBSERVATIONS ON THE MECHANISM OF FEEDING.

Many detailed observations were made on the mechanics of the feeding process in *Dileptus* in the hope of ascertaining the nature of the power of choice which *Dileptus* has been shown to possess. A few of them, illustrating the various factors involved in the feeding process, are described below.

A. Euglena.

In making observations on the capture and ingestion of *Euglena*, a single starved dileptus was isolated in a minute drop of water on a glass slide. To this another small drop containing many euglenæ was added. The reactions were observed under a magnification of about 350 diameters. In numerous observations it was found that whenever a euglena came in contact with any part of the oral surface of the proboscis of the dileptus, it at once become motionless (Fig. 2, *a*), and remained so for a longer or shorter
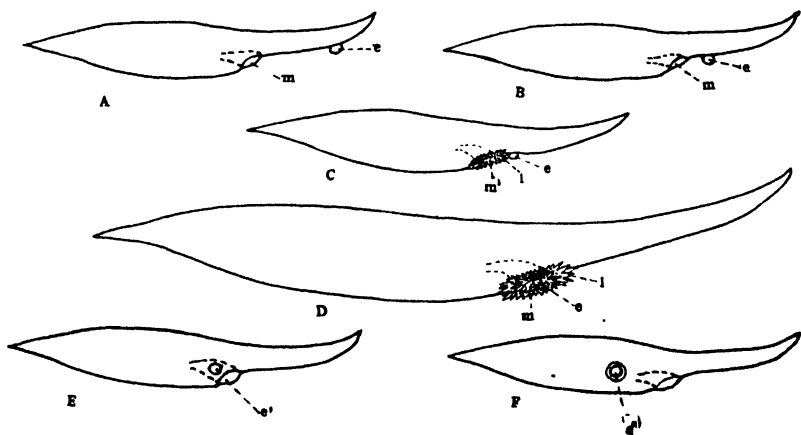
Fig. 2. Diagrammatic sketches illustrating the process of feeding. *A-F*, successive stages in process of ingesting *Euglena*. *e*, euglena paralyzed by trichocysts; *m*, mouth; *m'*, mouth with protruding lips (*l*); *e'*, euglena being engulfed; *e''*, euglena in food vacuole.

period, depending upon various conditions. It then reacted negatively with great vigor, and simultaneously or occasionally after a very brief latent period, it bulged out in the center and went through the typical euglenoid contractions with great vigor, sometimes holding the contracted form for two or three minutes. If, immediately after the first reaction, it failed to get out of the oral current which is continuously produced by the band of cilia on either edge of the flattened surface of the proboscis, it was carried to the mouth and engulfed (Fig. 2, b). If, on the contrary, the first reaction carried it outside the influence of the oral current, it began after a short interval to show some activity and soon recovered, unless it again came in contact with the proboscis. If this occurred, it was almost always carried to the oral region and engulfed.

The process of engulfing was quite extraordinary. Whenever the oral current carried a euglena to the mouth, the gullet, apparently owing to mechanical stimulation, protruded so that a mass of viscous protoplasm was exposed (Fig. 2, c). If any particle came in contact with this it adhered, and when this occurred the gullet was again drawn in carrying the particle with it (Fig. 2, d, e). In this process there was apparently some suction, for considerable water was always taken in with the solid particles (Fig. 2, f).

These observations were repeated on many favorable occasions, and, furthermore, are substantiated in the main by a similar observation recorded by Wrzesniowski ('70). Referring to the capture of a *Stylonychia* by *Dileptus,* Wrzesniowski says, "it tries by means of its proboscis to bring it down into its occasionally wide open mouth, whereupon the *protruding lips* seize so firmly upon the captured little animal that the latter is bitten in two." My observations agree with only the first part of this quotation; the idea of biting is quite contrary to the results of any observations which I have made.

B. ROTIFERS.

In the observations on feeding on rotifers described below, nine specimens were added to a small amount of water containing about twenty starved dilepti. Within two minutes all the rotifers were attacked. The dilepti appeared to sense (?) the rotifers while still

at a distance at least equal to their own length. Sometimes as many as two or three of these ciliates were seen to gather around and attack a single rotifer. In these attacks the dilepti usually failed to capture the rotifers. Only in one instance was a rotifer actually observed to be captured and eaten. Although the feeding process rarely culminated successfully, this experiment afforded observations which are very instructive, as the following indicate.

In these observations, each dileptus was continuously swinging its proboscis back and forth, and at the same time revolving on its longitudinal axis. Thus it struck the rotifers, now with the aboral side, now with the oral side of the proboscis, and the corresponding differences in the reactions of the rotifers were most striking. The rotifer in question is one which attaches itself quite securely to the wall of the dish. It also elongates and contracts from time to time without changing its location. When the aboral side of
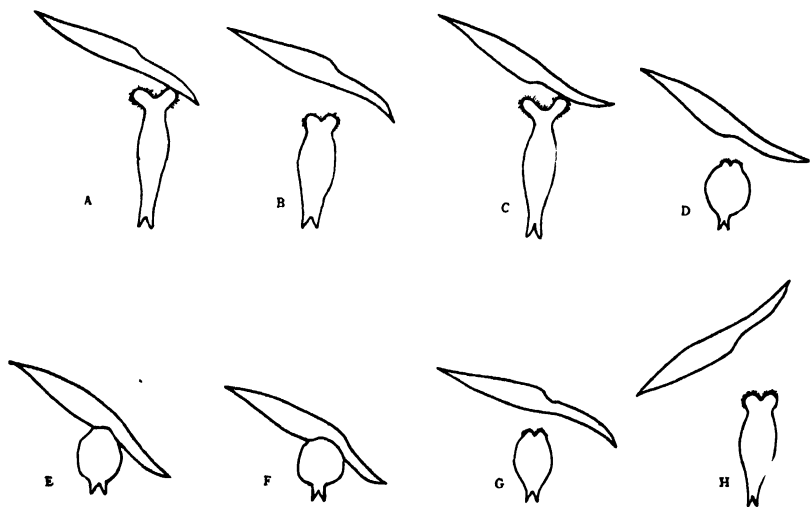
FIG. 3. Sketches illustrating the effect of *Dileptus* on rotifers. *A–H*, successive stages. Note differential effect, as seen by degree of subsequent contraction, of contact with aboral surface of proboscis as seen in *A* and *B*, and of contact with oral surface of proboscis as seen in *C* and *D*. For further explanation see text.

the proboscis of a dileptus came in contact with an elongated rotifer it contracted but slightly (Fig. 3, *B*), if at all, but when the oral surface of the proboscis struck the rotifer it contracted completely

and vigorously, and often remained thus contracted for some little time (Fig. 3, *D*). The hungry little ciliates still persisted and an occasional individual was sometimes seen to succeed in getting the head of an attached rotifer far into its oral opening (Fig. 3, *F*), but a sudden contraction on the part of the attached rotifer invariably resulted in freeing the captive.

Thus when a rotifer comes in contact with the oral surface of the proboscis of a dileptus its reaction is extremely vigorous, while if the aboral surface of the proboscis touches the same rotifer little, if any, reaction is observed. The reaction resulting from contact with the aboral surface of the proboscis is just such as would be expected from a sight mechanical stimulus, but the violent reaction observed whenever the oral surface comes in contact with the rotifer is clearly of an entirely different nature. This difference must be in some way related to the difference between the oral and aboral surfaces of the proboscis. The essential difference between these two is the fact that the former contains trichocysts, while the latter does not. The difference in the reaction is probably, therefore, related to the action of the trichocysts.

C. Colpidium.

The observations on *Colpidium,* like those on *Euglena* described above, were made under high magnification. In one of the many experiments two starved dilepti were isolated in a single drop of water and a smaller drop containing numerous specimens of *Colpidium* was added. The latter were so numerous that they were continually coming in contact with various parts of the dilepti. Some, consequently, frequently came in contact with the oral surface of the proboscis, as well as with various regions of the surface of the body. It was very apparent that those which came in contact with the oral surface of the proboscis were the only ones seriously affected. Whenever a *Colpidium* came in contact with this surface of the proboscis it at once became motionless and remained so for a very brief interval (Fig. 4, *A, a*). Then it suddenly became very active and swam away rapidly. Very often, however, with only a part of its body, for the part which came in contact with the proboscis bulged out and seemed to increase in volume, somewhat comparable to that which takes place when water

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is added to gelatine, but very much more rapidly. This mass was usually constricted off from the remaining part (Fig. 4, *B*, *b*), sometimes immediately, sometimes later. If this occurred immediately, the portion constricted off was carried to the gullet by ciliary currents and engulfed. Otherwise this portion was dragged along for a longer or shorter period by the active portion of the *Colpidium* (Fig. 4, *c*, *e*). Most of the *Colpidia* which were injured in this way soon died. Only a few of those which were isolated survived, and these had apparently lost only a small portion of their cytoplasm.
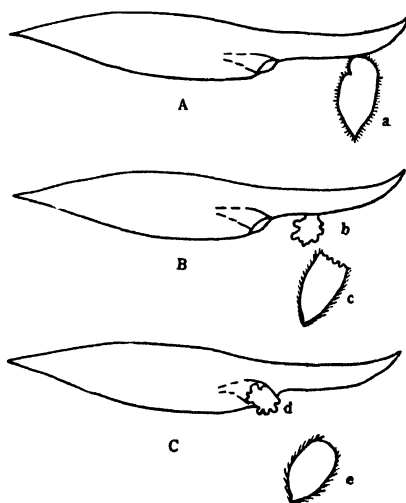
FIG. 4. Sketches illustrating the effects of the trichocysts of *Dileptus* on the infusorian *Colpidium*. *A*, *B*, and *C*, successive stages in the process of feeding. *a*, colpidium (motionless after contact with oral surface of proboscis); *b*, portion of colpidium (apparently cytolyzed and separated from major portion, *c*); *d*, cytolyzed portion being engulfed by dileptus; *e*, major portion of colpidium swimming rapidly away.

It seems evident from many observations like the above that it is the trichocysts of *Dileptus* which function primarily in the capture of *Colpidium*. The colpidia are first momentarily paralyzed and then excited to vigorous reaction, but as a rule only after a part of their cytoplasm has been in some way altered so that it disintegrates and *Dileptus* is enabled to feed on it in the same way that it does on other non-motile material.

The results presented seem to indicate that in *Dileptus* there are

two distinct processes involved in feeding: (1) The capture of food and (2) the ingestion of food. In feeding on motile organisms both processes are involved, but when feeding on non-motile substances only the latter process is involved. When feeding on motile forms the prey is first paralyzed, and in many cases where this is only temporary the cytoplasm of the prey is locally seriously affected. In some forms that part of the protoplasm affected is constricted off, while in other forms the entire organism is affected. In this non-motile condition the prey is carried passively by the ciliary current to the buccal cavity and there ingested as described above. The trichocysts are evidently the structures which enable *Dileptus* to capture living prey and make the feeding process of this organism so complicated. The remaining part of the paper will be devoted to observations and discussion as to their nature and function.

5. TRICHOCYSTS

The nature and function of trichocysts has long been a debated question and even today most authors admit that we know very little about them except in the case of one or two organisms which have been studied very extensively, and even here there is much controversy.

Mitrophanow ('04) maintains that the trichocyst consists of a viscid fluid contained in a cavity in the ectoplasm, whence it is expelled by a sudden contraction of the ectoplasm and stiffens to form a solid thread under the action of the water medium.

Schuberg ('05), however, denies this and maintains that the unexploded trichocyst is a spindle-shaped body with a fine hair-like process at its outer end which reaches to the pellicle, and that when it explodes this material forms into a fine thread-like sharp-pointed rod, often showing a cap-like swelling at one end.

Calkins in 1901 maintained that there are only two types which have been definitely made out—a rod-like form as in *Loxophyllum* and a spindle-shaped form as in *Paramecium*. He also stated that "when protruded from the body they are apparently of the same size and shape as when within the ectoplasm." In 1910, however, the same author wrote concerning trichocysts in general (p. 27), "when the organism is irritated the contents of the capsules are

thrown out with considerable force and the poison which they contain is strong enough to paralyze any single-celled opponent."

Minchin ('12) maintained that "the nature and mechanism of (the peculiar) trichocysts still remains to be explained," but described as typical those forms found in *Paramecium* and *Frontonia*.

Concerning the function of trichocysts there seems to be even less known than there is about the structure. Jennings ('06) wrote that trichocysts "are usually supposed to be weapons of defense, but whether they really serve for defense seems questionable," and suggested that their discharge may be only an expression of injury—"a purely secondary, even pathological phenomenon, like the formation of vesicles on the surface of an injured specimen."

Mast ('09), however, showed clearly that in *Paramecium* the trichocysts have a definite protective function. He observed that the trichocysts of *Paramecium* are discharged in response to injury, produced by *Didinium,* and that as soon as these trichocysts come in contact with the water they form a mass having a firm jelly-like consistency which serves to force the enemy back mechanically, and frequently results in setting the victim free. Calkins ('10, p. 27) says that "sometimes they are used as weapons of offense as well as protective organs," and in another place he describes predaceous protozoa as "usually armed with offensive organs in the form of trichocysts which may be shot out from the surface of the body or carried javelin-like at the extremities of projectile tentacles."

A. Observations on the Nature and the Function of the Trichocysts of *Dileptus.*

Numerous experiments and observations were made on *Dileptus* to ascertain, if possible, the function as well as the structure of the trichocysts, all of which, as previously stated, are located on the oral surface of the proboscis. A description of a few of the more illuminating of these experiments will follow, but before considering these we may briefly recall a few of the results of the observations on feeding which have a bearing on this subject.

Euglena, it will be recalled, is paralyzed as soon as it comes in contact with the oral surface of the proboscis, and after a short

latent period shows characteristic signs of injury. The violent contraction of the rotifers on every occasion when they come in contact with that portion of *Dileptus* provided with trichocysts gives definite signs of their effect. The observations on *Colpidium* show that the trichocysts not only paralyze this organism, but produce a cytolytic effect upon the protoplasm of the prey.

The following observations are presented in order to show more specifically the precise manner in which these trichocysts function.

a. Effect of Trichocysts on Paramecium bursaria.

In making observations on the action of the trichocysts of *Dileptus* on *Paramecium bursaria,* a single starved dileptus was isolated and added to a small drop of water on a slide containing four specimens of *Paramecium bursaria.* Nearly all the water was then drawn off, after which a cover-glass ringed with vaseline was applied. Two of the paramecia were lost, but the remaining two and the dileptus were confined in so small an amount of water and were so much compressed that they could move only very slowly, and never more than their own length from the others. Consequently all reactions could be observed very accurately. The dileptus, although so compressed that it was more than three times its normal width, continued to rotate on its longitudinal axis and its proboscis was consequently thrown from one side to the other. On several occasions the posterior end of the dileptus came in contact with one of the paramecia, making small indentations in it without any noticeable reaction on the part of the latter. When, however, it slowly reversed its position and the oral surface of the proboscis came in contact with the paramecium, a sudden discharge of trichocysts from the paramecium was observed, so dense as to force the dileptus away. The latter continued to rotate slowly, all the time removing the barrier of trichocysts by means of its ciliary action. The next time only the aboral surface of the proboscis came in contact with the paramecium and no reaction resulted. The third time the proboscis struck the paramecium it was at a slightly different spot and another discharge of trichocysts resulted from the latter. After some little time this again was cleared away, and a fourth attack occurred at about the same spot as the first, this time with an entirely different result. The paramecium

reacted much more violently than previously and at the point of contact a noticeable bulging of the protoplasm occurred. The next attack was at a new spot, with the characteristic discharge of trichocysts. But the following one was at approximately the same spot as the preceding. The protoplasm this time bulged out and formed a large protuberance, even some of the zoöchlorellæ flowing out into it. After half an hour four such protuberances were observed, in all of which it was evident that the pellicle of the paramecium had given way at one small spot, and that the mass of protoplasm which flowed out formed a protuberance with only a narrow connection with the interior. After repeated attacks the paramecium disintegrated.

The other paramecium, meanwhile, had slowly moved toward the dileptus, and this afforded an opportunity to repeat the observations just described. The results obtained in these observations were essentially the same as those obtained in the first observation. The second paramecium, however, appeared to react more vigorously and it consequently escaped more of the attacks than the first, with the result that at the end of half an hour it had only two protuberances, whereas the first, as previously stated, had four. Apparently each attack on the part of the dileptus was just as powerful at the end of the experiment as it was at the beginning.

b. Effect of Trichocysts on Stentor cæruleus.

Perhaps the most instructive, at least the most spectacular, experiment concerning the action of the trichocysts of *Dileptus* is one which can be performed very simply as follows: A dozen or more dilepti are starved for two days; a large blue stentor is then introduced, and the scene of a veritable barbecue is soon presented. The dilepti collect about the stentor and can be seen to strike the latter with their proboscides (Fig. 5). The surface that comes in contact with the stentors in this reaction does. not appear to be purely accidental, for it was observed that the oral surface of the proboscis came in contact almost without exception. At the point of contact the pellicle of the stentor gives way momentarily (Fig. 5, *D*, *d*), and a globular mass of protoplasm is extruded. This mass is soon constricted off (Fig. 5, *F*, *b*), and the wound apparently heals over at once, while the extruded protoplasmic mass is

readily ingested by the dileptus (Fig. 5, *G, b*). Thus, now here, now there, the stentor gives up part of its protoplasm and each part is eaten by the little dilepti, which sometimes more than treble in size after feeding on this organism. Meanwhile the stentor
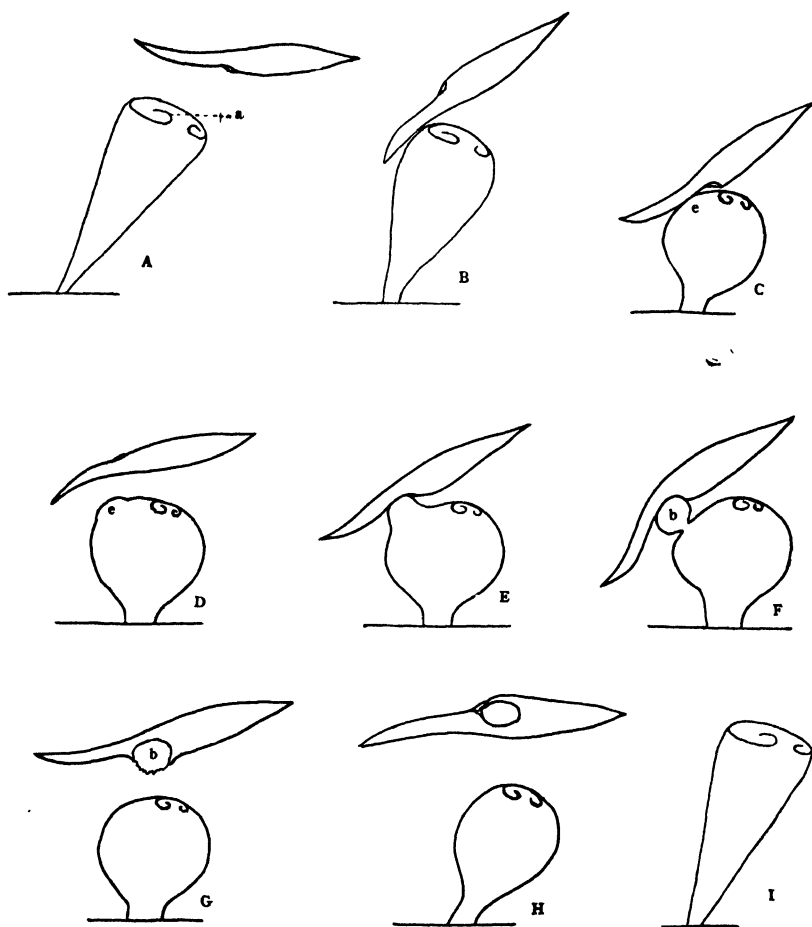
Fig. 5. Sketches illustrating effects of trichocysts of *Dileptus* on *Stentor cœruleus*. *A–I*, successive stages. *A*, normal stentor with epanded peristome (*a*). When stimulated as by contact with aboral surface of dileptus, the stentor contracts slightly as shown in *B*. If the oral surface of the proboscis comes in contact (*C*) the stentor contracts vigorously and its protoplasm in the region of contact (*e*) soon protrudes, *D, E,* and *F*. The injured area (*e*) soon gelates and the protruding mass (*b*) is constricted off, frequently being ingested by the dileptus (*G*). The stentor remains contracted for some time, but eventually, unless repeatedly attacked, it expands completely (*I*) and appears to be entirely normal.

continually shrinks in size, and unless it escapes before it is too badly injured it dies.

The trichocysts of *Dileptus* evidently affect the ectoplasm of *Stentor* and, as in the case of *Colpidium* previously described, result in a cytolytic action on the surface of the prey at the point of contact. This results in an outflowing of the inner protoplasm until the injured surface can again gelate in some manner, resulting in a new "pellicle."

If the observations just described are made under high magnification, it can be seen that the proboscis of dileptus never comes in actual contact with the body of the stentor. They are always separated by a space, at least equal to the sum of the lengths of their respective cilia. This would lead to the conclusion that the trichocysts of *Dileptus* are discharged through some little distance—that is, they are thrown out with some force.

c. Effect of Trichocysts on Paramecium aurelia.

Dileptus is normally unable to injure *Paramecium aurelia* in any way, but in one experiment several paramecia were seriously injured by two dilepti. In two instances, which were carefully observed, the paramecia appeared to be completely paralyzed, although only momentarily, immediately upon coming in contact with the oral surface of the proboscis. When the proboscis touched the paramecia they reacted vigorously and swam away, but not before they were injured. It was observed that they became much deformed soon after the attack, doubling on the point that had been injured to such an extent that they assumed the form of a horse shoe. One of these paramecia was attacked a second time while in this semi-quiescent condition and was successfully engulfed. The other one was isolated on a hollow ground slide and after about an hour began to swim about, gradually losing its deformity. On the following day it appeared to be normal. The ectoplasmic pellicle of *Paramecium aurelia* is probably of such a nature that it prevents any cytolytic action resulting from the trichocysts. The injurious effect of these structures seems to be due to the production of a definite wound at the point of contact.

d. Effect of Trichocysts on Spirostomum.

When a spirostomum is attacked by a dileptus it contracts vigorously as soon as "stung" (Fig. 6, A, B). This usually produces a violent reaction which serves to get it out of reach of the dileptus
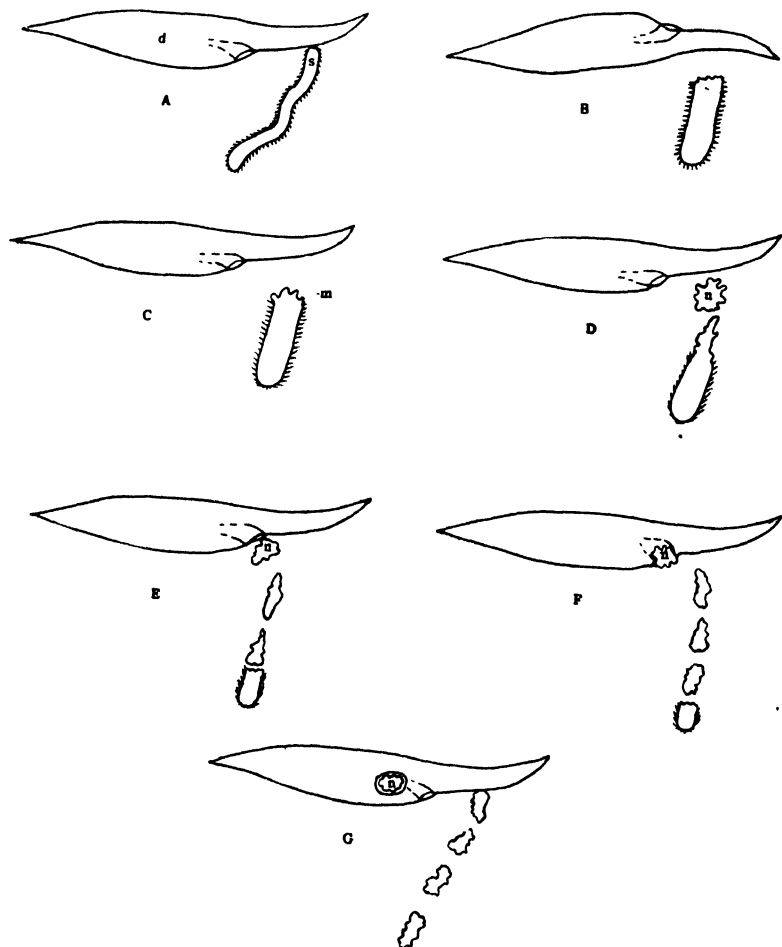
FIG. 6. Sketches illustrating the effect of trichocysts of *Dileptus* on *Spirostomum*. *A–G*, successive stages in process of feeding. When dileptus (*d*) comes in contact with spirostomum (*s*) as in *A*, the latter contracts vigorously and remains momentarily motionless (*B*). Cytolysis begins at area of contact (*m*) and as the spirostomum reacts negatively, swimming rapidly away, the cytolytic process continues, as in *D*, *E*, and *F*. Meanwhile dileptus has engulfed one or more masses of the disintegrating spirostomum (*n*) as shown in *D–G*.

and thus prevents a second attack. If it is attacked a second time, the result is extensive disintegration of the protoplasm around the point of contact (Fig. 6, *C, m*). If this point is located at one end of the spirostomum, the opposite end swims rapidly away, leaving behind a trail of disintegrating protoplasm (Fig. 6, *E–G*). When any part of the protoplasm of this organism begins to disintegrate, the cytolysis, once begun, progresses rather rapidly until the whole organism has disintegrated. This effect is in contrast with that obtained in *Stentor,* in which an attack produced only local and partial disintegration. Apparently the protoplasm of *Spirostomum* does not possess the power of gelation as observed in *Stentor,* and thus the cytolytic action continues until the organism is disintegrated (Fig. 6, *G*).

The results obtained in numerous other observations made on ' various other organisms are all in harmony with those which have been described. All these observations seem to show conclusively that the trichocysts discharged by *Dileptus gigas,* first temporarily paralyze the prey, then produce a period of increased activity in the nature of a negative reaction on the part of the prey, and simultaneously effect a cytolytic action at the point of contact.

B. OBSERVATIONS ON THE STRUCTURE OF THE TRICHOCYSTS OF *Dileptus.*

Numerous specimens of *Dileptus* were fixed during various stages in the process of feeding and many different methods of fixation and subsequently staining were employed in an attempt to ascertain the structure of the trichocysts. Before they are discharged the trichocysts can be clearly seen in all properly stained specimens (Fig. 7). They are found, as previously stated, in a band on the oral surface of the proboscis. When stained they appear as elongated bodies (Fig. 7, *t*), which do not show any definite internal morphological structure, but appear to be more or less granular, and if stained at all, are always stained deeply. Fig. 7 shows the relative number, size, and shape of the trichocysts as seen in $4\,\mu$ sections (*A–D*), and in a total mount (*E*). In favorable specimens they can be seen in the living animal, where they appear as colorless, rather transparent bodies which change shape as the animal twists and turns. They have been seen to become

almost spherical in shape when under pressure from the cover-glass.

In all my work it has been impossible to observe any structures or formed elements of any kind which could be identified as tricho-cysts or their contents outside the body of *Dileptus*.   The safranin
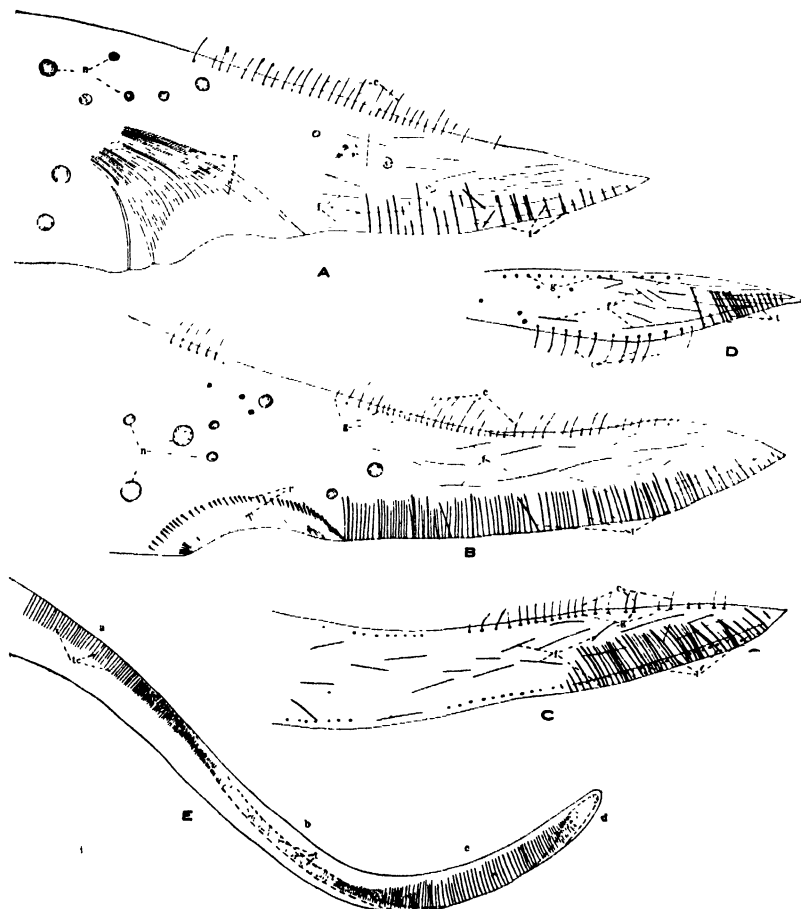
FIG. 7. Camera lucida drawings from preparations fixed in Schaudinn's fluid and stained with Fe-Haem. (*A–D*) or with acid borax carmine (*E*). *A, B, C,* and *D* are 4 μ serial sections of a proboscis. Note size, shape, and number of trichocysts (*t*); the pharyngeal rods (*r*); cilia (*c*); basal granules (*g*) "distributed nucleus," some of which are designated as *n*; and the con-tractile, fibrillæ (*f*). *E,* proboscis of dileptus, slightly twisted; from an entire mount. Note relative number and position of trichocysts (*t*), forming a band on the oral surface of the proboscis. Due to the turn in the proboscis, the trichocysts are seen from side view at *a* and *c* and from end view at *b* and *d*.

method of staining "intra vitam," which demonstrates so clearly the poisonous threads of the nematocysts of the common fresh-water hydra, was also tried, but gave only negative results.

We would maintain, then, that the contents of the trichocysts of this organism do not have a morphological structure after they are discharged, such as the trichocysts of *Paramecium* and *Frontonia* have. It would appear that the trichocysts of *Dileptus* are more like elongated sacs of toxic fluid, which collapse upon discharging the fluid.

If it is true that these structures found in the proboscis of *Dileptus* are bags filled with a poisonous fluid, it is evident that the term trichocyst (hair-sack) is not exactly applicable, and in order to be more exact the term toxicyst (poison-sack) might be employed.

C. SUMMARY OF OBSERVATIONS ON TRICHOCYSTS.

The foregoing observations and experiments show that the trichocysts of *Dileptus* are the structures which this organism employs in capturing food. They have the power to paralyze some organisms, to bring about the cytolysis of others, and to cause a vigorous reaction in almost all infusoria. Organisms like *Paramecium* and *Frontonia* are probably protected against the ordinary attacks of *Dileptus* by their own protective trichocysts. Organisms like *Euplotes,* which are provided with a lorica, form another class of infusorians which appear to be protected against the trichocysts of *Dileptus.* Certain species of *Stylonychia* are known to possess a heavy cuticle resembling a lorica, and it is perhaps for this reason that these organisms were but rarely observed to fall prey to *Dileptus.* The great majority of ciliates seem to fall prey to *Dileptus,* either owing to the paralyzing effect of its trichocysts or to the cytolytic action of these structures.

6. THE MECHANISM OF SELECTION OF FOOD IN *DILEPTUS*.

There appear to be two distinct mechanisms by which selection of food is brought about in *Dileptus.* (1) The rejection of inorganic particles, as shown in Tables I., II., and III., is evidently due to the effect of the physiological state, which serves to prevent the organism from ingesting more. (2) The purely chemical and

physical properties of the trichocysts of *Dileptus* seem to determine very largely the nature of the food which this organism ingests. If the trichocysts are able to bring about cytolysis of the protoplasm of an organism, or even to completely paralyze it for a time, that organism is "selected" as food. This relation between the protoplasm of the prey and the trichocysts of *Dileptus* is the important factor in determining whether or not *Dileptus* "selects" it as food.

In *Dileptus* the former mechanism seems to play but a small rôle. Because of its natural habits this ciliate deals almost exclusively with living organisms. As previously stated, *Dileptus* thrives only in "relatively pure" and quiet water in which there are but few inorganic particles in suspension. Its habit of continually swimming serves admirably to keep it off the substratum, and we can readily comprehend that motile organisms are almost the only substances from which it has normally to select. We can safely conclude that much of the power of selection of food in *Dileptus* resides in the peculiar properties of its trichocysts.

7. SUMMARY.

1. *Dileptus gigas* normally feeds on living organisms, but under certain conditions it ingests inanimate particles.

2. It discriminates between living organisms and inanimate substances, ingesting the former in large amounts, while the latter are only sparingly ingested.

3. *Dileptus* selects from among different kinds of organisms, eating some with great readiness, while others are rarely ingested.

4. It captures its prey by means of trichocysts which either paralyze the prey, *e.g., Euglena,* or bring about cytolysis of all or part of the protoplasm of the prey, *e.g., Colpidium* and *Stentor.*

5. The trichocysts are probably of a liquid nature, highly toxic, with specific cytolytic properties.

6. The trichocysts of *Dileptus* are used for the purpose of capturing food.

7. Selection of food in *Dileptus* depends on two factors: (*a*) The physiological state of the organism itself, which appears to determine whether a substance shall be ingested in large or small amounts, and (*b*) the chemical properties of its trichocysts, which

determine in large measure whether any living organism can or can not be successfully captured.

8. Specialized structures as, for example, the trichocysts of *Paramecium* and the lorica of *Euplotes,* serve as protection against the attacks of *Dileptus.*

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BIOLOGICAL BULLETIN

THE GROWTH OF THE PAINTED TURTLE.

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As Agassiz (1857) pointed out, the growth of turtles is exceedingly slow. After comparing turtles of different sizes, Agassiz concluded that the eastern painted turtle (*Chrysemys picta*), after the eggs from which it hatched were laid, attained about the following lengths after one year: 26.5 mm.; 2:42; 3:51; 4:54; 5:59; 6:66; 7:72.5; 8:74; 9:77; 10:80; 13:92; 24:121. He affirms that this turtle does not lay eggs until it has attained an age of ten to eleven years. Lucas (1922) states that there are authentic records of tortoises that have lived to be one hundred and fifty years old. Barney (1922) has given a very careful account of the growth and breeding of the diamond-back terrapin when reared under cultural conditions in pens. He found that when domestic terrapins are fed during the winter, egg production occurs as early as the fourth year of age, but usually begins in the fifth or sixth year. Terrapins may reach a length of 130 mm. to 150 mm. in four years when fed in winter, and in six to seven years when allowed to hibernate. The maximum growth in length recorded for one year was 81 mm., and for two years 104 mm.

Since 1919 the writer has had opportunity to study the growth of the western painted turtle.[1] At various times, 406 turtles were marked with aluminum tags and immediately released in University Bay, Lake Mendota. These were measured when they were released and were caught at intervals and measured again. In this way the rate of growth was determined. The portion of the bay behind the bar, where the turtles were studied, is nowhere more than 1.5 meters in depth, has a soft, muddy

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[1] Ruthven's (1912) *Chrysemys belli* Gray and *C. cinera* (Bonnaterre) appear to intergrade in this locality, but a majority of the individuals resemble the former.

TABLE I.

LIST OF RECORDS OF GROWTH OF TURTLES.

Abbreviations refer to months, from January to December, in the following order: J, F, M, Ap, My, Ju, Jl, Au, S, O, N, D, and indicate when each turtle was tagged and released.

Time Elapsed.			Length.		Gain.
Years.	Months.	Days.	Begin.	End.	
0	11	18	Jl 40.0	62.5	22.5
0	0	17	Jl 42.5	51.0	7.5
0	0	16	Jl 47.5	52.0	4.5
1	9	22	S 51	84.0	33.0
1	9	29	S 51	85.0	34.0
0	0	19	Jl 54	58.0	4.0
0	1	4	Jl 54	62.2	8.2
0	11	18	Jl 54	73.3	19.7
2	2	9	Jl 54	84.0	30.0
0	11	20	Jl 58.0	77.5	19.5
1	9	15	S 59	77.0	18.0
1	11	6	S 59	83.0	24.0
0	1	11	Ju 60	68.3	8.3
0	0	27	Jl 60	61.0	1.0
0	0	5	Jl 60	60.5	0.5
0	0	7	Jl 60	61.0	1 0
0	2	11	Jl 60	73.3	13.3
1	2	4	Jl 62	87.5	25.5
0	11	18	Jl 64	82.0	18.0
0	11	18	Jl 65	78.0	13.0
0	1	4	Jl 65	73.0	8.0
2	11	14	S 67	94.4	27.4
0	1	4	Jl 68	71.3	3.3
1	0	2	Ju 69	83.2	16.2
0	11	11	Jl 70.5	90.8	20.3
1	1	26	Jl 70.5	97.0	26.5
1	1	16	Jl 74	85.0	11.0
0	0	19	Jl 77	80.5	3.5
0	0	23	Jl 77	83.0	6 0
1	2	5	Jl 77	96.0	19.0
0	11	17	Jl 78.0	98.0	20.0
1	2	10	Jl 78	98.0	20.0
0	11	13	Jl 81.0	89.7	8.7
3	11	19	Au 82.0	95.8	13.8
1	5	16	F 87.0	91.0	4.0
0	2	18	Jl 89.5	91.0	1.5
0	11	17	Jl 89.5	92.3	2.8
1	2	21	Ju 90.0	108.2	18.2
0	3	3	Ju 90	·94.5	4.5
0	7	14	My 91.0	92.0	1.0
1	2	4	My 91.0	106.0	15.0
1	9	27	S 95.0	97.3	2.3
0	1	3	Ju 97.0	98.0	1.0
1	0	3	Ju 97.0	99.6	2.6
1	2	0	My 99.0	101.5	2.5
1	0	6	Ju 99.0	102.0	3.0
1	1	5	My 100.0	103.5	3.5

Table I, *Continued*

Time Elapsed.			Length.		Gain.
Years.	Months.	Days.	Begin.	End.	
0	11	23	Jl 101.0	110.2	9.8
1	0	2	Ju 101.0	111.0	10.0
1	2	4	My 103.0	106.7	3.7
2	2	10	My 103.0	109.7	6.7
2	11	22	S 103.0	113.5	10.5
0	1	0	Jl 103.0	103.0	0.0
0	10	18	Ju 104.0	107.0	3.0
1	11	27	S 105.0	108.9	3.9
2	0	6	S 105.0	109.5	4.5
1	2	11	My 105.0	110.0	5.0
1	2	0	Jl 105.0	110.0	5.0
1	1	25	My 108.5	111.0	2.5
1	9	20	S 112.0	119.0	7.0
1	2	7	My 112.0	112.2	0.2
1	1	5	My 112.0	114.0	2.0
0	11	18	Jl 113.0	123.2	10.2
0	11	14	Jl 113.0	115.0	2.0
1	2	11	My 114.0	118.5	4.5
1	2	4	My 116.0	117.3	1.3
1	0	6	My 118.0	120.0	2.0
1	2	4	My 120.0	123.0	3.0
0	11	10	Jl 121.0	128.7	7.7
1	1	22	My 121.5	123.0	1.5
1	2	4	Jl 127.0	130.5	3.5
0	9	0	Ju 127.3	133.5	6.2
0	0	16	My 129.0	129.0	0.0
2	1	4	Au 130.0	137.0	7.0
4	0	6	Au 130.0	139.0	9.0
2	1	4	S 130.0	136.0	6.0
0	2	11	Jl 131.0	131.5	0.5
0	9	10	Jl 131.0	131.5	0.5
1	1	27	My 132.0	132.0	0.0
2	2	4	My 132.0	135.5	3.5
1	2	6	My 134.0	139.0	5.0
0	1	1	Jl 140.0	140.0	0.0
2	2	3	Jl 140.0	144.0	4.0
1	2	26	Ju 140.0	143.3	3.5
1	2	0	My 142.0	144.0	2.0
1	1	20	My 142.0	142.0	0.0
0	11	13	Jl 148.5	150.0	1.5

bottom, and maintains a vigorous growth of aquatic plants. An abundance of food was therefore available.

The records are summarized in Tables I. and II. It will be noted that some individuals grew very rapidly during a few days, and that other individuals of about the same size increased little or not at all during a considerable period of time. Such differences are probably correlated with the shedding of the dermal plates of the shell, growth being rather rapid immediately after the plates are lost.

TABLE II.

GROWTH OF TURTLES OF DIFFERENT LENGTHS.

Length in *mm*.	Number of records.	Average Rate of Growth; *mm*. Per Year.	Estimated Average Weight; Grams.	Estimated Average Weight Increase; Grams Per Year.	Percentage of Increase.
40– 50	2	32.7	19	13.8	73
50– 60	10	17.0	36	11.1	31
60– 70	12	16.7	50	12.7	25
70– 80	8	19.0	68	15.9	25
80– 90	3	4.2	106	5.2	5
90–100	11	6.0	134	8.5	6
100–110	13	3.5	170	5.7	3.3
110–120	8	3.1	233	6.3	2.7
120–130	6	4.2	243	8.2	3.4
130–140	8	1.5	310	3.4	2.4
140–150	6	1.6	362	3.9	2.7

Table II. shows that a turtle nearly doubles its length and weight during the second year of its life. After twelve years it would be about 135 mm. long and the growth rate would have decreased to about one thirtieth of that during the first two years. An ordinary adult turtle measuring 150 mm. in length is, using the data here presented as a basis for computation, about twenty-five years of age (since the eggs from which it hatched were laid). The largest turtle measured from Lake Mendota was 170 mm. in length. It was perhaps fifty years of age.

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A NOTE ON THE TOXICITY OF ACIDS FOR MOSQUITO LARVÆ.

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Interest in the resistance of mosquito larvæ to various physical and chemical reagents has been largely, if not solely, directed toward the discovery of practical methods for the eradication of the mosquito. A large amount of literature dealing with this phase of the subject is now available. The effects of various solutions of salts on mosquito larvae have also been rather extensively investigated and it has been found that the animals are able to withstand rather high concentrations of pure salt solutions (Mac Fie (1), Chidester (2), Sen (3), Sharma (4), etc.). It has recently been pointed out by MacGregor (5) that mosquito larvæ are able to live and develop in extremely high concentrations of acid, *e.g.*, acid of $P_H = 4.4$. In view of these last mentioned observations, it was thought advisable to test the toxicity of a series of acids of different concentrations for mosquito larvæ and the present paper embodies results obtained from such experiments.

The larvæ used in all experiments, *Culex pipiens*, were obtained in large numbers from small, stagnant pools usually found in uncovered containers. The entire culture as found was brought into the laboratory and tests carried out at the same time and with the same group of animals, all of which had presumably been under identical conditions. Both young and old larvæ were used and differences due to age noted. The animals were removed from the cultures by means of a wide-mouth pipette and transferred to a syracuse watch glass with as little of the culture medium as possible. In this way a large number of larvæ could easily be obtained for use in each experiment. Fifty to sixty animals were used in testing the effect of any concentration of reagents. The chemicals used (10 c.c.) were put into covered syracuse watch glasses; the larvæ were quickly injected into the solution and then observed until dead under a binocular micro-

scope. The fatal exposure was taken at that time when approximately one half of the larvæ were killed, *i.e.*, when movements of the heart and alimentary canal ceased. Many cultures were used in experiments and slight variations in their resistance were shown but in the following only average results will be given. It is, however, of some interest to note that in practically all the cultures from which larvæ were obtained the hydrogen ion concentration showed them to be neutral or slightly alkaline in reaction (P_H = 7.0–7.4). The chemicals used were, hydrochloric, acetic, oxalic, butyric, salicyclic and carbonic acids and mercuric chloride.

FATAL EXPOSURES IN MINUTES TO DIFFERENT STRENGTHS OF ACIDS (TEMP. 25° C.).

Normality of Acid.	HCl.	Oxalic.	Salicyclic.	Butyric.	Acetic.
0.5	9.5	....	....	....	....
0.2	42.0	....	....	....	....
0.1	74.0	39.0	....	72.0	191.0
0.01	293.0	52.0	48.0	1440.0	1440.0+
0.001	1440.0+	1440.0	1200.0	1440.0+	1440.0++
0.0001	....	1440.0+	1440.0+	....	....

From the above table showing the length of life in minutes of ·larvæ in various strengths of acids it is evident that the animals are able to withstand abnormally high concentrations of acids for rather long periods of time. This remarkable resistance of mosquito larvæ is more strikingly shown when compared with that found for other forms—*e.g.*, Honda (6) found that the free-living nematode *Rhabditis elegans* withstood 0.01 normal HCl for 60 minutes, *Daphnia* for 23 minutes, tadpoles for 12 minutes and paramecium for 1 minute. (Personal communication to be published in *Journal of Experimental Zoölogy.*) MacArthur (7) found that Planarians are killed in a very short time by exposure to HCl of P_H 2–4.5. It has also been found by the author (8) that cysts of *Colpoda* withstand 0.001 N HCl for a strikingly long time.

That the hydrogen ion concentration is not necessarily the only factor in the toxicity of acids for larvæ is shown by comparing the effects of a saturated solution of CO_2 in H_2O of a P_H of approximately 3.7 with a solution of HCl of the same P_H value.

Larvæ in the CO_2 solution become motionless almost at once and the movements of heart and alimentary canal also quickly cease, while in HCl of the same P_H value they are apparently unaffected for over 24 hours. The more rapid penetration rate and mode of action of CO_2 as pointed out by Jacobs (9), doubtless account for the differences observed in the effect of the two reagents.

It is also of much interest to know in what manner the acids kill the animals, whether they enter the chitinous covering or enter by the mouth or anus through the alimentary canal. By using pupae, which are known not to eat nor to have external openings as in the larvæ, it is found that the acids do not kill them for many hours, considerably in excess of the lethal exposure for larvæ. From this fact it seems reasonable to assume that the larvæ are killed by the entrance of the reagent orally rather than cutaneously. The present discussion, however, deals primarily with the resistance of the animals to the reagents rather than with their mode of killing. Younger and smaller larvæ, and these doubtless have thinner chitin, are killed somewhat more quickly than older individuals.

The general order of toxicity of the acids used for the larvæ is, salicyclic > oxalic > HCl > butyric > acetic. This series is strikingly similar to that found by Haas (10) for plants, by Collett (11) for protozoa and by Bodine (8) for cysts of *Colpoda.*

FATAL EXPOSURES IN MINUTES TO DIFFERENT PERCENTAGES OF $HgCl_2$
(TEMP. 25° C.).

Per Cent. $HgCl_2$.	Time in Minutes.
0.05	305.0
0.10	155.0
0.50	48.3
1.00	26.5
2.00	20.0

Mercuric chloride in various concentrations was used and here again the resistance of the larvæ is of considerable interest. The above table shows the effect of different percentages of this salt. Honda (6) found that the free-living Nematode *Rhabditis elegans* withstood 0.05 per cent. $HgCl_2$ for 60 minutes; *Daphnia,* 25 minutes and tadpoles, 5 minutes. Mosquito larvæ are about 5 times as resistant to $HgCl_2$ as the most resistant form used by this author. Sen (3), with other species of mosquito larvæ (*Ste-*

gomyia albopicta) found that the animals were killed at once in a
0.1 per cent. HgCl₂ solution. This difference in length of time
of fatal exposure to this concentration from the present result is
doubtless due to the differences in resistance of the two species
as well as to the end point taken in the experiments. Sen evidently
used cessation of body movement while in the present investi-
gation cessation of movements of heart and alimentary canal
were taken as the end point.

SUMMARY.

1. Mosquito larvæ (*Culex pipiens*) were found to be extremely
resistant to rather high concentrations of various acids.

2. The order of toxicity of the acids used is, salicylic > oxalic
> HgCl > butyric > acetic.

3. The chemicals seem to penetrate the animal orally and not
cutaneously.

4. Animals withstand rather high concentration of HgCl₂,
considerably in excess of that found for other organisms cited.

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THE AMŒBOID MOVEMENT OF DISSOCIATED SPONGE CELLS.[1]

PAUL S. GALTSOFF.

INTRODUCTION.

In 1907 H. V. Wilson discovered a very interesting case of regeneration in siliceous sponges from dissociated tissue cells. The same phenomenon was observed in Hydroids, *Alcyonaria*. and Asteriæ (Wilson, 1911), in fresh water sponges (K. Müller, 1911) and in calcareous sponges (Huxley, 1920).

Twelve years before Wilson's discovery Roux (1895) described a similar phenomenon. He found that the blastomeres of the frog egg, artificially separated at an early stage of segmentation, and placed in water a short distance apart, slowly approached one another until they came into contact. Roux called this phenomenon cytotropism to correspond with other tropisms known in the scientific literature. He regarded cytotropism as a special case of chemotropism. This opinion was based on theoretical considerations as no experiments were made to prove it.

The term cytotropism although not definitely accepted has been used in various text books and scientific papers. Apparently the sponges with their ability to form the conglomerates from the dissociated cells afford the best opportunity for a study of this phenomenon.

Wilson and Müller (l.c.) made their studies on the regeneration of sponges after dissociation only after the aggregates began to form. Prior to this they made no exhaustive study, and merely stated that the separated cells coalesce and form aggregates. They did not study the amœboid movement which leads to the coalescence of cells and which is the main purpose of the present investigation. The work was started in 1920 at the Marine Biological Station at Sebastopol (Black Sea) and continued in 1921 at Woods Hole and in the Zoölogical Laboratory of Columbia University.

The writer desires to express his gratitude to Dr. F. R. Lillie, Director of the Marine Biological Laboratory at Woods Hole, for accomodations there and to Dr. T. H. Morgan, of the Department of Zoölogy, Columbia University, for the courtesy of extending to him laboratory privileges.

Species Used for Experiments.

The coalescence of cells after their dissociation has been observed in many species of sponges. It is probable that all sponges possess this ability, but the formation of dense conglomerates and the power to regenerate a new organism occur only in a few forms. The species tested by the writer are as follows: Black Sea sponges—*Halichondria grossa* Schm., *Petrosia clavata* B. Cor., *Reniera densa* Bowerb., *Reniera informis* Schw., *Esperella lorenzii* Sch., *Kowalevskiella gracilis* Swarc., *Spongelia* sp., *Sycon* sp.; Woods Hole sponges—*Microciona prolifera* Verr., *Cliona celata* Gr., and *Grantia* sp.

Upon dissociation the cells of these species are able to coalesce and to form aggregates, but the grade of the formation varies. The best forms are *Reniera informis, Reniera densa, Petrosia coriacea*, and *Microciona prolifera*. The formation of aggregates in these species requires less time, the aggregates are more strongly attached to the substratum and they quickly transform themselves into new sponges.

Unfortunately many of the microphotographs, drawings, and other data collected by the writer at Sebastopol were lost owing to unavoidable circumstances and therefore the present work deals chiefly with the experiments made on *Microciona prolifera* at Woods Hole.

Amœboid Movement of Dissociated Cells.

The suspension of dissociated *Microciona* cells, obtained by squeezing the sponge through bolting silk No. 20, consists of three classes of cells, each of which can be easily recognized. The most abundant are the archaeocytes, nonspecialized, reddish cells about 8 microns in diameter and loaded with granules. Two kinds of these cells can be discriminated: the endoplasm of the first contains red pigment granules, to which the red color of the sponge is mainly due; the second contains in addition to a less

abundant red pigment many dark yellow-greenish granules. Both forms are able to put out rounded or sometimes elongated pseudopodia and to display an active amœboid movement.

The second class consists of spheroidal dermal cells differing in size from 8 to 3 microns in diameter. They move very slowly, putting out short rounded pseudopodia.

To the third class belong the collar cells or so called choanocytes. They are partially dedifferentiated having lost their collars but still possess a long slender flagellum which continues to vibrate for at least three hours.

The process of reunion of the dissociated elements of the sponge tissue consists of following stages; sinking of the cells and adhesion with one another, adhesion to the bottom, amœboid movement and coalescence of cells, movement and coalescence of aggregates. The process of the formation of the aggregates can be easily observed in a microaquarium or in a hanging drop on a hollow slide. In both cases the globular aggregates are formed within three to four hours, the difference being that in the microaquarium the aggregates strongly adhere to the glass, while in a hanging drop they remain floating.

Among the different tissue elements of sponge the archaeocytes are the most active; some of them may send out pseudopods within a few minutes after the sponge was squeezed. They begin to move as soon as they come in contact with the bottom. The coalescence may even occur while the cells are still in suspension.

The character of the amœboid movement of both yellow and red archæocytes is the same; the cells put out large hyaline pseudopods and creep in different directions. The inner granuloplasm of the cells appears to be more viscous than their surface hyaline layer. This can be easily observed when two cells come in contact with one another. When two archæocytes touch one another their external hyaloplasm spreads out from both sides of the line of contact and flows round their bodies. Sometimes one can notice how the hyaloplasm is pressed away from the contact line between the cells and surrounds their bodies.

Often the archæocytes lose small drops of hyaloplasm which remain behind them indicating the route of the cell. Another archæocyte passing the same way may wipe out these drops which coalesce with its protoplasm.

On coalescence the inner granular protoplasm of the archæo-cytes remains unmixed; the granuloplasm of various cells occupies a definite portion of the aggregate, while the hyaloplasm forms a common mass surrounding the whole group.

By pressing the coverglass under which the aggregates are lying or by violently shaking a dish containing aggregates the cells can be separated after which they are able to coalesce again.

The amœboid movement of the archæocytes is irregular; some of them move actively while the others remain motionless or creep very slowly over the bottom. There is no visible difference between sluggish and active archæocytes. Frequently after being immobile for 60 or 80 minutes the archæocyte becomes active and starts to move rapidly.

In many cases when two archæocytes coalesce the aggregate remains motionless for a few minutes after which it starts to move in another direction. The coalescence with a small dermal cell or with a choanocyte does not disturb its movement; it con-tinues to move as if there was nothing in its route.

The small dermal cells move very slowly or not at all. The choanocytes do not display an amœboid movement but are able to displace themselves by means of their active flagella.

The ectoplasmic layer of the isolated archæocytes is fluid and sticky. The cells easily adhere to different objects which come in contact with them. The adhesiveness of their protoplasm can be strikingly seen in a suspension containing starch grains. Each aggregate formed in such suspension is surrounded with a ring of starch grains. This occurs because the starch grains ad-here strongly to the cells; as the aggregates move and turn in different directions they become surrounded with grains with which they accidentally come in contact. When moving the cells are able to push along or to carry various foreign bodies which they meet in their route. In one observation a small aggre-gate was found strong enough to push a group of five starch grains each of which was larger than the aggregate itself.

The formation of the aggregate from dissociated tissue cells is due to the motility of certain archæocytes and to the stickiness of their outer layer of protoplasm. The various cells are uniform-ly distributed in the suspension and over the bottom of the dish; each active archæocyte moves back and forth over a definite

area and therefore cleans up the corresponding portion of the bottom, collects all cells lying in its route and finally forms an aggregate.

A well formed 24-hour-old aggregate of *Microciona* has the form of a ball; its surface is smooth and a thin hyaline membrane can be noticed on its periphery. Under unfavorable conditions the aggregates are irregular in form and fail to form a membrane. They are then unable to undergo futrher transformation and to regenerate into a new sponge.

DIRECTION OF CELL MOVEMENT.

There arises a question whether the approaching and the coalescence of the cells are due to a special kind of chemotropism or "cytotropism" or whether their movement is chaotic and their approach is a matter of a pure accident. If there be a directive force one may expect that the cells will move towards several others which form the centers of the attraction. In other words one ought to be able to detect a definite directive movement.

The simplest way to study the direction of cell movement is to draw contours of cells at definite intervals and then project all the outlines on one surface and in this way to reconstruct the paths of the cells. Such an investigation was made with a camera lucida and a combination of the Zeiss objective E and eyepiece 6 (X 625). A microaquarium was filled with a dilute suspension of Microciona (1 gram of sponge per 200 c.cm. of sea water); a sufficient number of paper sheets were placed on the table at the level of the microscope stage and were pierced at two points. The holes made by the punctures enabled one to put the sheets exactly upon one another after they had been removed from the table. Special precautions were made not to disturb the lower sheets when the upper one was removed. The sketches were made every two minutes and often every minute. The observations lasted from 40 to 190 minutes. After this period the movements are so slow that the continuance of the observation was unpracticable. The temperature of the water during the observations varied from 19° to 21° C. The observations were repeated many times with different colonies of *Microciona;* in all cases the character of the movement was the same, the dif-

ference being only in the velocity and in the duration of the movement.

The examination of the paths of various cells shows that their movement is not at all directed towards one another or towards the group of cells. No one cell could be found which acts as a center of attraction for other cells. The coalescence occurs only when one moving cell happens to touch another one, and to stick to its outer layer. The path of the archæocyte is an irregular winding line; a typical case is shown in Fig. 1. The movement of this archæocyte was followed for 168 minutes. The points where coalescence with other cells occurred are indicated by crosses; the arrows show the direction of the movement. Other cells in the same field of view moved very slowly and passed only few microns; the aggregate was formed exclusively through the activity of the archæocyte.
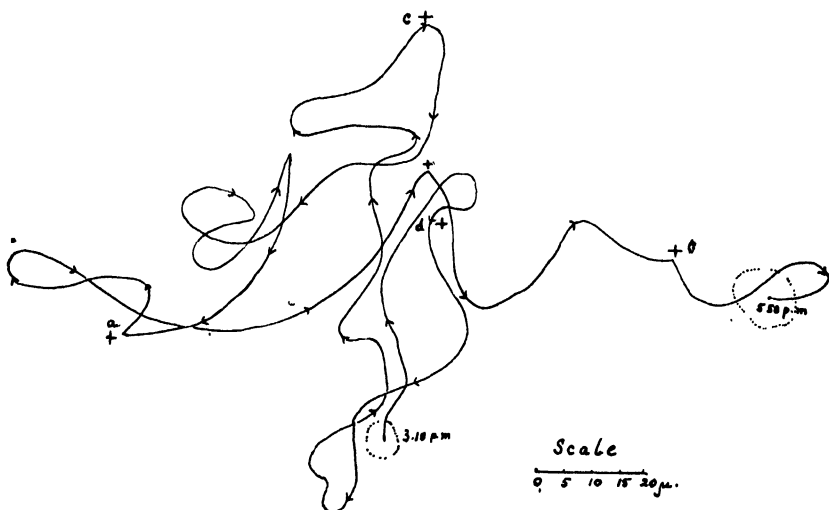
FIG. 1. The path of the archæocyte of *Microciona*; the line representing the movement of the center of the cell. The observation was made with camera lucida, Zeiss obj. E, and eyepiece 6. The outlines were drawn each two minutes.

This archæocyte travelled back and forth in different directions, approaching certain cells from which it withdrew. It coalesced first with the small dermal cell *d*, with the chaonocyte *c*, and with two archæocytes *a* and *b* all of which happened to lie in its route.

The velocity of the movement of the archæocyte is subject to

many fluctuations. Even a fast moving cell sometimes stops and after a short period of rest resumes its movement. The retardation as a rule takes place when the cell changes its direction. The average velocity of an archæocyte measured in ten different experiments varied from .6 to 3.5 microns per one minute; the maximum velocity was as great as 20.0 microns per one minute. This maximum velocity was observed only twice. The distance travelled varied from 64 to 185 micrones and active movement lasted from 40 to 168 minutes.

THE BEHAVIOR OF THE DISSOCIATED CELLS IN A COMPOUND SUSPENSION.

The cells of two different species of sponges mixed together coalesce only with cells of their own species. This ability of cells to discern the more foreign elements can be easily observed when two sponges of different colors are used. Wilson (1910) pointed out that the cells of *Microciona* mixed with those of *Lyssodendrix* and *Stylotella* form different clumps each apparently composed of the cells of same species; the clumps could be recognized by their natural colors. The same was found by the writer when a mixed suspension of *Reniera informis*, violet, Reniera densa, gray, and of *Microciona*, red, and *Cliona*, yellow, were tested. The aggregates formed in such emulsions were a violet, gray, red and yellow.

It is quite easy to distinguish the colors of the aggregates but as the color of a single cell is very slight it is impossible to distinguish the species while the cells are in suspension. In order to find how complete is the separation of the cells in a mixed suspension, one of the sponges before the experiment was fed with carmine, the other, with Chinese ink. The red and black granules ingested by the cells serve as definite marks indicating the species.

The suspension contained the cells of *Reniera informis* fed with carmine and that of *Reniera densa* fed with Chinese ink. The resulting aggregates lying close together consist exclusively either of carmine or of ink laden cells. The same occurs in a mixture of *Microciona prolifera* and *Cliona celata*.

Coalescence with the cells of another species never occurs even when the cells are artificially pressed together by centrifuging a

mixed suspension. Examining after 24 hours such a clump of *Microciona* and *Cliona* one can find that in a common yellow mass of Cliona cells the Microciona had formed globular aggregates with marked membranes separating them from cells of the other species.

The formation of the aggregate in a mixed suspension does not go so completely as in a pure one; the aggregates are smaller and correspondingly more numerous than in the control dish. They are unable to undergo further transformation.

The coalescence of the dissociated tissue cells of sponges is apparently the same phenomenon as occurs in the extravasated blood cells of Arthropoda (Tait, 1918, Leo Lòeb, 1920). In both cases the mechanical or chemical changes in the environment of the cells lead to its amœboid activity and to formation of aggregates of separated cells. In sponges the archæocytes *i.e.*, non-specialized elements, form aggregates which are able to regenerate a new organism. In blood cells the process does not go so far; the amœbocytes join in clumps and under favorable conditions can form a certain kind of tissue (L. Loeb). There is no indication of chemotropic or cytotropic stimuli in both cases and no such hypothesis is required to explain the results.

Conclusions.

The coalescence of separated sponge cells is the result of two factors: first, amœboid activity of the archæocytes, second a specific physical property of cell protoplasm which enables the cells to coalesce when they come into contact.

The coalescence of cells of two different species never occurs apparently because the physical properties of the protoplasm of the various species are different.

So-called cytotropism or a special kind of chemotropism does not exist in the cases studied.

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OBSERVATIONS AND EXPERIMENTS ON EUGLE-NOIDINA IN THE DIGESTIVE TRACT OF FROG AND TOAD TADPOLES.

ROBERT W. HEGNER.[1]

On a number of occasions during the past decade the writer has observed living flagellates of the *Euglena* type in the intestinal and rectal contents of frog tadpoles. They were always consider-ed merely accidental inhabitants that had been ingested with the food of the tadpoles and were either immune to the digestive juices and were on their way through the intestine or had not, yet succumbed to digestion. The observations and experiments described below, however, furnish evidence (1) that these flagel-lates are of widespread occurrence among the tadpoles of a numb-er of species of frogs and toads, (2) that they are normal inhabi-tants of the intestine and rectum of tadpoles in the same sense that the better known protozoa, such as *Opalina*, are, (3) that they persist in starved tadpoles for many days, even after *Opalina* has disappeared, (4) that they retain their green color for a considerable period within the body of the tadpoles, (5) that they can be transferred in the trophozoite stage from one species of tadpole to another with food material, (6) that they differ in structure from any free-living or parasitic Euglenoidina heretofore described, (7) that they do not grow and multiply easily under ordinary culture conditions, (8) that certain free-living species of Euglenoidina are digested by tadpoles that do not digest the entozoic species, and (9) that certain tadpoles that were heavily infected with Euglenoidina did not become full-grown and undergo metamorphosis.

1. *A Comparison of Normal Tadpoles of Rana pipiens with Tadpoles Containing Large Numbers of Species A.*—The first series of observations, recorded in Table I. indicate that the

euglenoid flagellate that I shall call Species A*, is a constant in-habitant of the tadpoles of *Rana pipiens* from Ithaca, N. Y., and that it may be a factor in the failure of certain specimens to grow and undergo metamorphosis normally. So far as I have been able to learn, this species has not been described although Alexeieff (1912) noted what may have been specimens of this form in the intestines of tadpoles. It is cylindrical and elongate with blunt anterior and posterior ends and decidedly metabolic. It ranges in length from 35 μ to 45 μ and in breadth from 4.8 μ to 6.4μ. The average length of ten specimens was 39.7μ and average breadth, 5.3 μ. There are three flagella almost as long as the body and about 50 green chromatophores, oval or circular in outline, lying in a single layer near the surface of the body. The nucleus is spherical and situated near the center of the body. Near the anterior end is a large spherical reservoir open-ing to the outside through a cytopharynx. At one side of the reservoir is the red stigma. Figs. 1 to 3 are of three specimens drawn with the camera lucida at a magnification of 1600 diame-ters. The specimen in Fig. 1 was living when drawn, in Fig. 2, killed and stained with iodine, and in Fig. 3, fixed in Schaudinn's fluid and stained by the iron-hæmotoxylin method.

The tadpoles were collected in artificial ponds at the fish hatch-ery of Cornell University at Ithaca, N. Y. My attention was directed to their study by Dr. G. C. Embody, who had noted the small size of specimens in one pond as compared with those in a neighboring pond only six feet distant. These ponds were about 4 feet square and 18 inches deep. One was well filled with algæ and the other was almost free from vegetation. Egg masses of *Rana pipiens* had been placed in these two ponds by Dr. Embody at approximately the same time, about April 20. The tadploes were collected seven weeks later (June 8). In Tables I. and II. are presented data regarding the differences (1) between the two sets of tadpoles and (2) between their rectal contents. The following points may be noted.

1. *Size*.—(Table I.) Although the two groups of tadpoles

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* After this paper was written I learned that Dr. D. H. Wenrich had also spent the summer of 1922 studying this organism. His work was largely devoted to its morphology and cultivation. He has given it the name Euglenamorpha hegneri. (Wenrich, 1923).

studied were of approximately the same age at the time they were examined they differed greatly in size. Those in the pond without algæ being on the average approximately twice as long in both
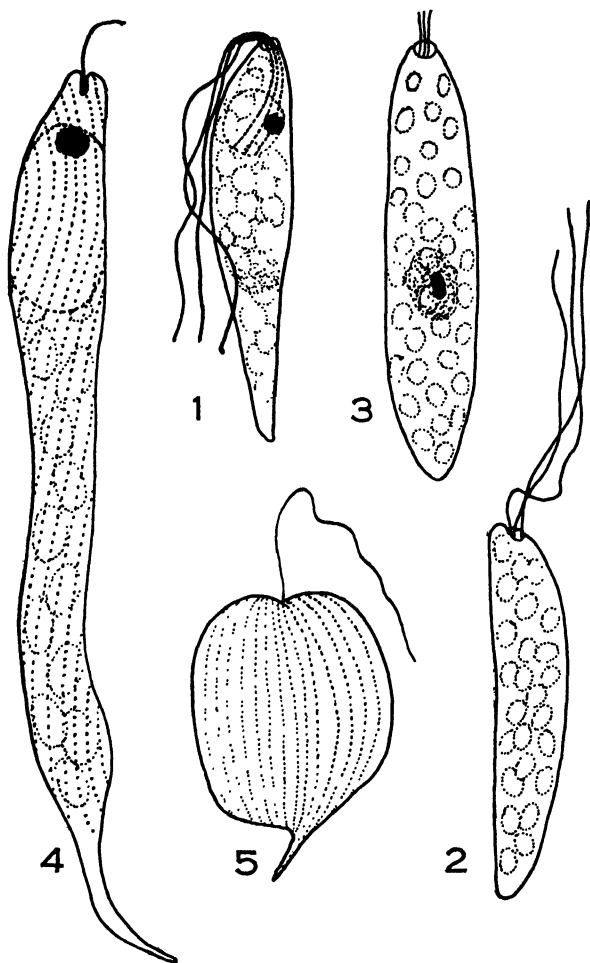
FIG. 1. A living specimen of a euglenoid of Species A showing characteristic shape, three flagella, reservoir, stigma, chromatophores and nucleus. X 1600.

FIG. 2. Species A as seen when stained with iodine. X 1600.

FIG. 3. Species A fixed in Schaudinn's solution and stained with iron-hæmotoxylin. X 1600.

FIG. 4. A living specimen of Species B showing flagellum, reservoir, stigma, and chromatophores. X 1600.

FIG. 5. A living specimen of *Phacus* found in the intestine of *Rana pipiens* tadpoles. X 780.

body and tail and in total length as the others. Every one of the "normal" tadpoles was larger than any one of the "dwarfs."

2. *Length of Intestine.* (Table I.) A high correlation between body size and length of the intestine was found. In almost every case the larger the body the longer was the intestine. The average length of the intestine of tadpoles from the alga pond was less than half that of the other tadpoles.

TABLE I.

MEASUREMENTS OF BODY, TAIL, INTESTINE AND RECTUM, IN MILLIMETERS, OF TEN SPECIMENS EACH OF "DWARF" AND "NORMAL" TADPOLES OF
Rana pipiens.

	Length of Body.		Length of Tail.		Total Length.		Length of Intestine.		Length of Rectum.	
	Range.	Average.	Range.	Average.	Range.	Average.	Range.	Average.	Range.	Average.
10 "Normals"	10–15	13.4	17–24	20.8	27–39	34.2	75–165	127.3	5–20	12.1
10 "Dwarfs"	6–11	8.1	8–15	11.2	15–25	19.3	30–92	58.3	3–10	5.7

3. *Length of Rectum.* (Table I.) The rectum of the tadpole is coiled in such a way that exact measurements are difficult to make. Those in the table are only approximate, but they show that in the "dwarfs" the rectum was only about half as long as in the "normal" tadpoles.

4. *External Evidences of Metamorphosis.*—All of the "normal" tadpoles exhibited rudiments of hind limbs. These measured from 2.5 to 0.5 mm. in length. Only one of the "dwarfs" possessed rudiments of legs and these were only 0.25 mm. long. The former had therefore progressed further in metamorphosis than the "dwarfs."

5. *Internal Evidences of Metamorphosis.*—Shortening of the intestine occurs during the metamorphosis of tadpoles. In judging this character, however, one must take into account the size of the tadpole. The shorter length of the intestine of the "dwarf" tadpoles is probably due to the small size of the animal rather than to a more advanced stage of metamorphosis. In both groups of tadpoles the rectum was well differentiated.

6. *Character of the Contents of the Intestine.*—The intestines

of the two groups of tadpoles differed in appearance, the differ-
ence being due apparently to the presence of algæ in the dwarfs
and the absence of algæ in the normals. The intestines of the
latter were filled with very fine particles of mud mixed with
diatoms and minute organic debris; they were grayish in color,
of uniform thickness, and smooth in outline. The intestines of
the dwarfs, on the other hand, contained large pieces of algæ
which appeared to divide the contents into separate masses,
giving the entire intestine a patchy appearance; they were slightly
tinged with green, and had an irregular outline due to the irregular
distribution of the contents. The organisms noted in the intes-
tines of both sets of tadpoles included a very few normal inhabi-
tants of the rectum—*Opalina, Trichomonas,* and *Hexamitus;* at
least 60 per cent. of both sets were inhabited by *Giardia agilis;* ·
euglenoids were found in four of the dwarfs and one of the nor-
mals; and filamentous algae were present in considerable abund-
ance in all of the dwarfs but in only one of the normals.

7. *Character of the Contents of the Rectum.*—The contents of
the rectum of the normal tadpoles gave this part of the alimentary
canal a grayish appearance, but in the dwarfs the euglenoids
were so abundant that a distinct greenish color was produced.
It was difficult to count accurately the number of specimens of
the various organisms present. The method employed was to
mix thoroughly on a slide the entire rectal contents in one drop
of normal saline solution; spread this out under an 18 mm. square
cover glass and count the number of organisms in ten separate
fields in different regions of the slide using a 4 mm. objective and
a 4 × ocular. Averages for each tadpole were then computed.
Table II. gives the range and the average numbers of five different
organisms. *Endamœbæ* and other forms were encountered but
were not recorded. *Opalina, Trichomonas,* and *Hexamitus* were
present in every tadpole and no significant differences in number
were noted. The most conspicuous difference in the two groups
of tadpoles was the presence of large numbers of euglenoids and
of considerable amounts of filamentous green algæ in every one
of the dwarfs and the almost complete absence of these in the
normals. One or several euglenoids were found in the rectal
contents of six of the normals after long searching and a little
algæ was seen in one of them.

TABLE II.

COMPARATIVE NUMBERS OF SPECIMENS OF CERTAIN ORGANISMS IN THE RECTUM OF TEN SPECIMENS EACH OF "NORMAL" AND "DWARF" TADPOLES OF *Rana pipiens*.

.The numbers were obtained by counting those in ten fields with a magnification of 520 diameters.

	Opalina.		Tricho-monas.		Hexa-mitus.		Euglenoid Species A.		Fila-mentous Algæ.	
	Range.	Average.	Range.	Average.	Range.	Average.	Range.	Average.	Present.	Abun-dant.
10 "Normals"	0.2–0.5	0.44	8–32	15.7	15–80	35.7	1		1	0
10 "Dwarfs"	0.1–2.0	0.67	3–48	13.6	2–40	23.6	4–60	16.0	8	2

The euglenoids were thus almost entirely restricted to the rectum of the dwarf tadpoles. They seemed to be in excellent condition; the pigment spot was bright red; the chlorophyll bodies were a brilliant green; and swimming activities and metabolic changes were apparently normal.

8. *Conclusions.* This comparative study of 10 specimens each from these two sets of tadpoles leads to the following conclusions:

(*a*) The euglenoid, Species A, is a constant inhabitant of the rectum of the tadpoles of *Rana pipiens* obtained from an alga pond at Ithaca, N. Y., and a rare inhabitant of the rectum of tadpoles of the same species from a neighboring alga-less pond. Infection with *Opalina, Trichomonas,* and *Hexamitus* was about the same in both sets of tadpoles.

(*b*) Although of approximately the same age the tadpoles containing many euglenoids were only about one half the size of the other set; and were less advanced in metamorphosis. The presence of these euglenoids may have been a factor retarding growth and metamorphosis.

II. *Effects of Starving the Host on the Persistence of Euglenoids of Species A in Tadpoles of Rana pipiens.*—After a comparison was made between tadpoles of *Rana pipiens* one set of which contained euglenoids in abundance and the other set few or none, it was decided to keep infected tadpoles in the laboratory and examine them at intervals to see if the infection persisted for any

[1] One or several specimens were present in 6 of the 10 tadpoles.

considerable period and if any changes in number, stage in life history, color, etc., would take place. Table III. gives the dates

TABLE III.

NUMBER AND DISTRIBUTION OF EUGLENOIDS OF SPECIES A IN TADPOLES OF *Rana pipiens* COLLECTED JUNE 12 AND KEPT IN LABORATORY WITHOUT FOOD UNTIL DATE OF EXAMINATION.

For method of counting see text.

Date Examined.	Number of Days in Laboratory without Food.	Average Number per Field in Rectum.	Average Number per Field in Intestine.
June 17.	5	Abundant	Many
June 18.	6	8	24
June 19.	7	10	3
June 20.	8	23	Very few
June 21.	9	37	Very few
June 22.	10	19	Many
June 24.	12	22	Many
June 25.	13	Many	Many
July 4.	22	Very few	Many
July 7.	25	30	Very few

and results of examinations. One tadpole was used each day, the last one being examined on the twenty-fifth day. During this entire period the euglenoids persisted in the digestive tract, in numbers at least as great as in tadpoles examined on the date of collection. My method of counting (see p. 88) was not very accurate, but it seemed to me that the number of euglenoids was more numerous in tadpoles studied on later dates than at first. In the meantime the other protozoa common in the rectum of these tadpoles decreased markedly or disappeared entirely. No encystment was noted in any of the specimens and only one euglenoid was seen in division.

The euglenoids retained their normal free-swimming shape throughout the entire experiment, and were very active, swimming about by means of their flagella or undergoing rapid metabolic movements. No appreciable decrease was noted in the intensity of the green color nor in that of the eye-spot. This was probably due to the transparency of the ventral body-wall which allowed light rays to enter. A few days after the tadpoles were brought into the laboratory the rectum and intestine became almost free from food material and their contents could easily be seen through their walls. It was found that the euglenoids

were usually most abundant in the rectum (see Table III.), almost as numerous in the first 10 mm. of intestine adjacent to the rectum, and fewer in number throughout the rest of the intestine. They were not, as a rule, distributed throughout the intestinal and rectal contents, but could be seen swimming about between these contents and the wall. Often they occurred in large groups thus giving a patchy green color to the digestive tract that could be discerned with the naked eye.

That these euglenoids also persist in tadpoles in nature was proved by the examination of three specimens collected from the same pond on June 24, *i.e.*, 12 days after the first lot were taken. These specimens all contained numerous euglenoids in both rectum and intestine; some of the euglenoids seemed to have become paler in color. The larval period of *Rana pipiens* is from 60 to 80 days but neither the tadpoles collected on June 12 and kept in the laboratory until July 7 nor those collected on June 24 and kept in the laboratory until July 9 increased in size nor advanced in development during this time, although they were about 75 days old and should have been undergoing metamorphosis. The presence of euglenoids may have been a factor in this retardation of growth and development.

III. *Infection of Rana pipiens Tadpoles with Food Containing Species A*.—Tadpoles of *Rana pipiens* in which there were a very few specimens of Species A were collected on June 24, 1922, when about 9 weeks old. On the following day 5 of these were fed on the recta from ten tadpoles of the same species in which there was an abundance of Species A. Previous examination of tadpoles from the same lot as those from which these recta were obtained gave an average number of sixteen euglenoids per field (see Table II., "dwarfs"). The 5 experimental tadpoles immediately began to devour the recta and all of the latter had been eaten by the following day and no specimens of Species A could be found in the dish. Uninfected (normal) and infected tadpoles were examined at intervals of one, three, five, nine, and twelve days. The results obtained are given in Table IV. It is evident from the increase from 0.6 per field to 4.4 per field that Species A has increased in the experimentally fed tadpoles and that this increase is due to the ingestion of specimens contained in the recta used for feeding purposes. Since these specimens were

TABLE IV.

COMPARATIVE NUMBERS OF EUGLENOIDS OF SPECIES A IN THE RECTUM OF TAD-
POLES OF *Rana pipiens* USED AS FOOD AND IN CONTROL AND
EXPERIMENTALLY FED TADPOLES.

Character of Tadpoles.	Number of Specimens.	Numbers per Field of Species A in Rectum.	
		Range.	Average.
Dissected for feeding..........	10	4–60	16.0
Controls.....	5	0.2–0.9	0.6
Experimentally fed...........	5	2–7	4.4

probably all in the free-swimming stage it seems certain that infection of *Rana pipiens* tadpoles can be brought about by the ingestion of active trophozoites. Such a method of infection might occur in nature since tadpoles will feed upon the dead bodies of other tadpoles, but this is probably not the usual method since 100 per cent. of infection has been observed in entire schools of young tadpoles, and, of course, there must be a resistant stage for maintaining the race through the winter and for infecting the first tadpoles in the spring.

IV. *Can Euglenoids of Species A be Cultivated Outside of the Body of ths Tadpole?*—The data presented above indicate that the euglenoids of Species A are regular inhabitants of the digestive tract of *Rana pipiens* tadpoles. The questions suggested by these results are; (1) are these euglenoids restricted to this habitat or can they also maintain a free-living existence; and (2) can other euglenoids known to be free-living be colonized in the rectum and intestine of tadpoles of this species. Two methods of answering these questions were employed: (1) an attempt was made to cultivate Species A outside of the body of the tadpole, and (2) tadpoles were fed on freeliving euglenoids and their digestive tract examined on subsequent days.

Euglenoids of Species A remained alive and active for at least 48 hours inside of the digestive tract that had been dissected out of tadpoles and kept in a small dish in water. Specimens also remained alive for 72 hours in material from the rectum and intestine under a sealed cover glass. Specimens that were dissected

out of the digestive tract and placed in culture dishes did not live and multiply. This does not prove that they cannot maintain themselves outside of the digestive tract of the tadpole but indicates that they probably are restricted to an entozoic existence.

V. *Can Tadpoles of Rana pipiens be Infected with Free-living Euglenoids?*—It has been shown above that Species A can be transferred from one tadpole to another with the food, therefore if free-living species can live successfully in the digestive tract of these tadpoles it should be possible to bring about infection by including them with the food. On June 24, twenty-five tadpoles of *Rana pipiens* about nine weeks old were placed in a culture containing millions of small free-living euglenoids of a species possessing 2 short flagella, obtained from a large tub at the fish hatchery. In 18 hours these tadpoles had eaten every euglenoid in their medium. At this time five of these tadpoles and an equal number of controls were examined. The rectum of the experimental tadpoles was of a deep green color and the intestine also. Not a single specimen of the free-living euglenoids, however, could be found in any of these five tadpoles. The greenish color was due to minute chlorophyll bodies from 3 μ to 8 μ in diameter. These were the chromatophores of the disintegrated euglenoids. Chlorophyll bodies of this type were entirely absent from the five control tadpoles examined at the same time. In both experimental and control tadpoles there were present a few euglenoids of Species A and a few of a species to be described later as Species B. Experimental and control tadpoles were examined on the second day (5 specimens), fourth day (2), and eighth day (1). No euglenoids of the free-living type were discovered. The chlorophyll bodies gradually decreased in number. The conclusion reached is that an essential difference exists between euglenoids of Species A and those of this free-living type, the former being able to withstand the digestive juices of the host and to maintain themselves within the digestive tract, whereas this free-living species is unable to live in the same environment being killed and digested by the tadpole.

A second experiment was carried on at Baltimore during the month of September. Five tadpoles of the green frog that contained very few euglenoids were placed in a small amount of water in which there were thousands of a large reddish-colored

euglenoid. One tadpole that was examined the following day contained many living euglenoids most of which were rounded and quiescent but a few of which were extended though sluggish. Besides these euglenoids there were large numbers of euglenoid chromatophores present proving that many specimens had been broken down within the digestive tract. The feces of the remaining four experimentally fed tadpoles were found to contain living euglenoids and these in the course of the next two weeks must have passed through the digestive tract of these tadpoles and been reingested again and again. One tadpole was killed and examined four days after feeding, another 13 days after feeding and the last two 20 days after feeding. The first two of these contained living euglenoids in both intestine and rectum, but the other two, which were prevented from reingesting their feces for a period of 7 days were entirely free from euglenoids. These results show that the free-living euglenoids used in this experiment had a higher degree of resistance to digestion within the tadpole than those employed in the first experiment but were unable to maintain themselves for a period of 20 days within the digestive tract.

A third experiment of a similar type was carried out with euglenoids obtained from the bladders of the bladderwort, *Utricularia*. I am indebted to my colleague, Mr. Bruce D. Reynolds, for calling my attention to this form and obtaining material for me. Plants obtained from a pond on the campus of the Johns Hopkins University were well supplied with these euglenoids although the surrounding water was entirely free from them. The numbers of euglenoids counted in ten bladders ranged from 8 to 510 in each, with an average of 215. Euglenoids were dissected out of 90 bladders and placed in a dish of water with three tadpoles of the green frog. The total number of euglenoids in this dish was thus about 20,000. All of these had disappeared from the water by the following day and none could be found in the fecal material in the dish. One tadpole was examined after two days and the other two after three days. No euglenoids were found in any of them. This type of euglenoid, therefore, is unable to withstand the digestive juices of the tadpole, although resistant to the secretions within the bladder of the *Utricularia* plant.

VI. *Euglenoids of Species A in Toad Tadpoles.*—A large number of toad tadpoles were collected from one of the large ponds at the fish hatchery on June 12, 1922. Their average measurements were: total length, 20 mm.; body, 8 mm.; tail, 12 mm.; hind legs, 3 mm.; intestine, 47 mm.; rectum, 9 mm. They were kept in the laboratory in a flat dish without change of water but with the addition of fresh water from time to time to compensate for evaporation. Some of the tadpoles developed fore legs and acquired the characteristics of the young toad within a few days; these apparently had reached a stage when no more food was necessary to bring this about. In most of them, however, growth and metamorphosis were inhibited by the condition of starvation to which they were subjected. Specimens were examined at intervals with the results presented in Table V. The following

TABLE V.

NUMBERS OF EUGLENOIDS OF SPECIES A IN THE RECTUM OF TOAD TADPOLES
COLLECTED ON JUNE 12, 1922, AND KEPT WITHOUT FOOD.

For method of counting see text.

Date Examined.	Number Examined.	Number of Days in Laboratory without Food.	Range in Number of Specimens per Field.	Average Number of Specimens per Field.
June 28.........	10	16	1–12	5.9
July 11.........	3	29	4–6	5
July 13........	2	31	1–8	4.5

observations seem worthy of mention. (1) The incidence of infection with Species A was 100 per cent. (2) No encysted or dividing specimens were encountered. (3) Most of the euglenoids were swimming freely or undergoing metabolic movements; a few were spherical or pear-shaped. (4) The number of specimens was not diminished or increased by the starvation of the host. (5) Evidently Species A is a "normal" inhabitant of the rectum of toad tadpoles in this locality. (6) A decided decrease in the intensity of the green color of the chromatophores was noticeable in almost all specimens; some were pale green and others were almost colorless. This condition probably resulted from lack of light and may be contrasted with that observed in the case of specimens from starved tadpoles of *Rana pipiens*.

In the latter the abdominal body wall allows the light to penetrate to the intestine more easily than in the toad tadpole, which is characterized by the presence of dense black pigment. The nutrition of the euglenoids in the toad tadpoles thus becomes almost entirely by absorption whereas in the tadpoles of *Rana pipiens* it is still partly holophytic. It is interesting to note that Species A is sensitive to light, congregating on the side of the slide toward a north window and moving from one side of the slide to the other, a distance of 16 mm. in about 20 minutes, when placed opposite this window.

VII. *Infection of Toad Tadpoles with Species A by Association with Infected Tadpoles of Rana pipiens.*—Evidence was presented above that tadpoles of *Rana pipiens* can be infected with euglenoids of Species A by feeding them food containing active trophozoites, but this probably is not the method of infection in nature. Inasmuch as all tadpoles in certain ponds were found to be infected and none or a very few in other ponds, infection by association was suggested. To test this method the following experiment was carried out. A large number of toad tadpoles were collected on July 10. Five of these were examined on July 23 and found to be uninfected. Seven of the remaining tadpoles were then placed in a finger bowl with 7 tadpoles of *Rana pipiens* taken from a lot that were all infected. They were kept together for five days. Three of the toad tadpoles were still alive, three had recently died, and one had died previously and been removed. Each of the three living toad tadpoles, on examination, was found to contain large numbers of Species A in the rectum and a few in the intestine. No specimens were found in one of the dead tadpoles but a few were present in the other two dead tadpoles. At this time ten more toad tadpoles from the control lot were examined; eight of these were uninfected and one specimen of Species A was found after careful search in the rectum of each of the other two. These results prove that uninfected tadpoles may become infected by associating closely with infected tadpoles. The obvious way in which this is brought about is the escape of specimens from the rectum of infected tadpoles into the water and their ingestion by the uninfected tadpoles. This probably occurs in nature only when the tadpoles are closely associated. Toad tadpoles are very gregarious and might easily

infect one another even in a large pond. Tadpoles of other species are much less accustomed to congregate in numbers and hence would not transfer their infection unless confined to a small body of water.

VIII. *Species A in Tadpoles of the Green Frog, Rana clamitans.*—Euglenoids of Species "A" were encountered in very small numbers in tadpoles of the green frog. For example, several specimens were found in the rectum of each of four tadpoles that were collected on June 14 and kept in the laboratory without food until June 26. That these euglenoids may be taken in with the food of the tadpole and localized in the rectum is evident from the following experiment. One tadpole of the green frog, collected on June 14 was starved until June 28 and then fed the rectal contents of three toad tadpoles containing euglenoids. Two days later a considerable number (two per field) of Species A were found in the recutm of this specimen—a much greater number than had been found in any of the several hundred other green frog tadpoles previously examined. Furthermore most of these specimens were pale in color like those in the toad tadpoles at this time.

IX. *Specificity of Euglenoids of Species A.*—It seems probable from the observations and experiments recorded above that euglenoids of Species A are "normal" inhabitants of the recutm and intestine of tadpoles of the frogs and toads that inhabit freshwater ponds. Those living in different hosts may be specifically distinct but no evidence of this was obtained. Attempts to infect adult salamanders proved negative. Ten infected toad tadpoles were fed to a specimen of *Diemyctylus viridescens* on July 1. Four days later no traces of euglenoids could be found in the digestive tract of this specimen, indicating that this salamander cannot be infected with its food and that these euglenoids are probably unable to live in adults of this species. Another specimen of this species of salamander was placed in a dish containing millions of free-living euglenoids. Four days later, the intestine of this animal contained green chlorophyll masses, but no recognizable euglenoids.

X. *Euglenoids of Species B.*—A second type of euglenoid was noted in the rectum and intestine of tadpoles of *Rana pipiens* and *R. clamitans.* Euglenoids of this species, which may be

referred to here as Species B, probably were present in all of many *Rana pipiens* tadpoles collected when about 9 weeks old. They were recorded incidentally in 32 of them, although very little attention was devoted to them at the time. They were noted also in five large tadpoles of the green frog but were probably also of general occurrence in this species. Species B is a cylindrical, elongate species with a blunt anterior end and a posterior end terminated either by a short subacute tip or by a longer process which is slender, pointed at the tip and slightly curved. No free swimming forms were seen but most of the specimens were undergoing slow metabolic movements. Speci-,mens ranged from 99 μ to 128 μ in length and from 9 μ to 16 μ in breadth, with an average length of 110 μ and average breadth of 12 μ. A single short flagellum that moved too slowly to disturb the particles in the surrounding medium was observed in several specimens. Numerous disciform green chromatophores and a large reservoir and red stigma are present. The periplast is spirally striated and strongly punctated in some specimens. In many respects it resembles *Euglena spirogyra* Ehren. Fig.4 is a camera lucida sketch of a living specimen magnified 1600 diameters.

Euglenoids of Species B were never numerous in any tadpole. They were more often observed in the intestine than in the rectum. Their ability to live in these environments and their frequent occurrence indicate that they are normal inhabitants of the digestive tract of tadpoles. An attempt was made to increase their numbers by feeding the rectum and intestine of infected tadpoles to other infected tadpoles. Thus the digestive tracts of 22 tadpoles were fed to four tadpoles of *Rana pipiens* on July 4. Not all of this food was eaten and after 24 hours living active specimens of both Species A and Species B were observed within the walls of the intestines and recta offered as food. Specimens were still alive and active after 48 hours. One tadpole was killed and examined after an interval of three days (July 7) and the remaining three after six days (July 10) but no increase in the numbers of Species B could be ascertained with certainty. I believe there was an increase but the actual numbers were too small to determine the point definitely.

XI. *Species of the Genus Phacus.*—In a few of the tadpoles of

Rana pipiens a species of *Phacus* was noted that resembled in size and shape, *Phacus pleuronectes*. They were about 42 μ long and 32 μ broad, oval in shape with an uncinate posterior spike about 6.5 μ long, a longitudinal groove extending anteriorly from the posterior end, a longitudinally striated periplast, and a large number of green chromatophores, as indicated in Fig. 5. These organisms remained alive and active for 48 hours in intestinal contents that were sealed under a cover glass with vaseline.

XII. *The Effects of the Presence of Euglenoids on Other Intestinal Protozoa.*—No apparent effects on the number and distribution of other intestinal protozoa were noted due to the presence of Euglenoidina. There was, however, a change in the color of many of the opalinas. As a rule *Opalina* is almost transparent when studied with a compound microscope but whenever euglenoids were abundant many of them took on a greenish yellow tinge. This was true in the tadpoles of both *Rana pipiens* and the toad and was very noticeable in the tadpoles that were fed on free living euglenoids. Evidently nutrition in *Opalina* in the presence of chlorophyll bearing bodies is a process whereby some of this coloring matter is taken in thus causing a greenish tinge.

XIII. *The Parasitic Habit among the Euglenoidina.*—Only a few of the Euglenoidina have been described as parasitic in habit. Prof. L. B. Walton mentions two species of the genus *Astasia* in his monograph on the Euglenoidina (Walton, 1915) and has called my attention (in litt.) to other members of the order described by Alexeieff (1912) and Nieschultz (1922). *Astasia captiva* is endoparasitic in *Catenula lemnae*, and *A. mobilis* in the egg sacs and digestive tract of *Cyclops*—a condition that has led to the suggestion by Alexeieff that parasitic Sporozoa may have originated in this way. Nieschultz describes a species of *Astasia* from the digestive tract of a fresh-water nematode, *Nilopus gracilis*, but does not give it a specific name. Haswell (1907) reported the occurrence of a euglenoid inside the cells of a rhabdocœle Turbellarian. Alexeieff reports the discovery of small, living, active euglenoids, *Euglena* sp. and *Phacus longicauda*, in frog tadpoles and states that Brumpt also showed him specimens containing hundreds of *Euglenæ*. The green color and stigma of these were as bright as in free-living specimens. This, accord-

ing to Alexeieff, is a case of accidental parasitism which is a stage in the evolution of parasitism among the Euglenoidina. He accounts for the presence of these euglenoids by the mode of nutrition of the tadpoles, which engulf large quantities of debris of all sorts resulting in a thick, compact mass in the intestine. Organisms in the midst of such a mass might easily escape the action of the digestive juices, especially since these are greatly diluted, and as a result become gradually acclimated and finally facultative parasites.

XIV. *Summary and Conclusions.*—(1) Three species of Euglenoidina are described from the intestine and rectum of frog and toad tadpoles; all three species possess green chromatophores and bright red stigmas.

(2) A comparative study of two sets of tadpoles of *Rana pipiens* from adjoining ponds, one set much retarded in growth and heavily infected with Species A and the other of normal growth but lightly infected or not at all, indicates that the dwarfing of the former may have been due to the presence of euglenoids.

(3) Tadpoles of *Rana pipiens* infected with Species A were kept in the laboratory without food for 25 days and specimens examined at intervals. The infection persisted throughout this period without any marked decrease in the brightness of the green color of the euglenoids. Only one case of division was noted, and no cysts were found, the euglenoids remaining as trophozoites, free swimming and actively metabolic, throughout the period. The retention of the green color may have been due to the transmission of light through the almost transparent abdominal and intestinal wall. The rectum is the usual habitat of this species but the intestine is often invaded especially the first 10 mm. just anterior to the rectum. They are not mixed with the intestinal and rectal contents but move about between this mass and the containing walls. Infection was found to persist in tadpoles collected from time to time from the pond.

(4) Tadpoles of *Rana pipiens* containing very few specimens of Species A were fed on the intestines and recta of highly infected tadpoles of the same species. A great increase in the number of Species A in the experimentally fed tadpoles proves that infection with this species can be brought about by the ingestion of active trophozoites with the food. This, however,

is probably not the usual method of infection in nature, since a resistant over-wintering form of Species A probably exists by means of which the new broods of tadpoles are infected in the spring.

(5) Attempts to cultivate Species A outside of the tadpole failed and it seems probable that trophozoites are incapable of living and reproducing themselves in water outside of the host. Specimens were kept for several days in intestines and recta that had been dissected out and placed in water and also in rectal contents sealed under a cover glass. The latter proved to be sensitive to light congregating on the side of the slide toward a north window and moving from one side of the slide to the other, a distance of 16 mm., in about 20 minutes, when placed opposite this window.

(6) An attempt was made to infect tadpoles of *Rana pipiens* with three species of free-living euglenoids. The euglenoids were all ingested but none became colonized in the digestive tract. One of these euglenoids was taken from the bladders of *Utricularia* in which they were able to maintain themselves in spite of the secretions present there. The species that inhabit the intestine and rectum of the tadpoles therefore possess a resistance to digestive juices not present in free-living forms.

(7) All of a large group of tadpoles of the toad, *Bufo lentiginosus americanus*, were found to be infected with Species A, but another group of tadpoles of this species from another pond were not infected. Specimens of infected tadpoles were kept in the laboratory without food for 31 days and examined at intervals. The euglenoids persisted throughout this period. No apparent increase in numbers was noted and no division stages nor cysts were observed. There was no decrease in size. The organisms were free-swimming and actively metabolic. The green chromatophores, however, gradually became paler in color until the specimens were almost transparent. This loss of the green color was probably due to the failure of sufficient light to penetrate the deeply pigmented abdominal wall of the toad tadpole.

(8) Euglenoids of Species A were also found in the intestine and rectum of tadpoles of the green frog, *Rana clamitans*. They were not as numerous as in tadpoles of *Rana pipiens* or of the toad. One tadpole that was fed on the intestines and recta of three infected toad tadpoles became more highly infected, thus proving

that active trophozoites from the latter can be transferred to tadpoles of the green frog with their food.

(9) It is evident that euglenoids of Species A are regular inhabitants of the intestine and rectum of three species of tadpoles, *Rana pipiens*, *R. clamitans*, and *Bufo lentiginosus americanus*, and that they can be transferred from tadpoles of one species to those of another with the food. No specific differences were noted in specimens from different species of tadpoles.

(10) Euglenoids of Species B were present in tadpoles of *Rana pipiens* and *R. clamitans*, but were never very numerous. The intestine seemed to be more highly infected than the rectum. The specimens observed were as green as free-living species and contained brightly colored stigmas. Most of them appeared to be without flagella and either remained stationary except for metabolic movements or squirmed slowly from place to place. Attempts to increase the number present in one tadpole by feeding it infected intestines and recta of other tadpoles were not definitely successful.

(11) A species of *Phacus* resembling *P. pleuronectes* was observed in a few tadpoles of *Rana pipiens*.

(12) The presence of euglenoids had no apparent effect on other protozoan inhabitants of the digestive tract except in the case of certain opalinids which became yellowish green in color. · The advantages of the group of organisms dealt with in this paper as material for a study of the evolution of parasitism is obvious and the writer expects to continue work on the group with this object in view.

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BIOLOGICAL BULLETIN

THE AXIAL GRADIENTS IN HYDROZOA. V. EXPER-
IMENTAL AXIAL TRANSFORMATIONS IN
HYDROIDS.

C. M. CHILD.[1]

The hydroid stolon usually appears in nature as a basal out-growth readily distinguishable from the stem by its habit of growing in contact with surfaces rather than free in the water. Contact has often been regarded as a factor in determining its formation, but it has been observed by many investigators that in the reconstitution of isolated pieces of hydroid stems stolons may develop from that end of the piece which was origi-nally apical, as well as from the basal end, even when these ends are not in contact with solid surfaces. In some cases also it has been observed that isolated pieces of certain species develop only stolons which may later give rise to hydranths or may con-tinue to grow as stolons. Peebles (1900) states that this fre-quently occurs in pieces of *Hydractinea* and *Podocoryne* when they are left in dishes undisturbed without change of water. Loeb ('92) maintained that stolon formation in *Antennularia antennina* is determined by gravity, but Morgan ('01) and Stevens ('02, '10), while not denying the correctness of Loeb's conclusions, demonstrated beyond a doubt that other factors than gravity may be concerned. They were not able, however, to reach definite conclusions concerning the nature of these factors. Lund ('21) has shown that when isolated stem pieces of certain hydroid species are exposed to the electric current, stolons tend to arise at the end toward the cathode, hydranths at the end toward the anode. Various other data might be cited, but it seems fair to say that no one thus far has been able to discover a general physiological basis for the development of

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stolons in hydroids. The original polarity, gravity, contact, the electric current, light, have all been mentioned as factors concerned in determining the polarity of hydroids, but no one of these has been shown to be of fundamental or general importance as regards stolon formation.

The present paper is concerned with observations and experimental data which throw some further light upon this problem. These data were accumulated during four summers, 1917–1920, spent at the Puget Sound Biological Station, and I take this opportunity of expressing again my obligation to the Director for the facilities afforded.

MATERIAL AND METHODS.

Three hydroid species, *Bougainvillea mertensi, Obelia borealis* and *Gonothyræa clarkii*[1] were chiefly used, but a few experiments were performed with other species.

In the course of other work with hydroids it was observed that often in *Bougainvillea,* and occasionally in other forms, freshly collected stocks showed stolons in place of hydranths. Commonly these stolons were found in the basal one fourth to one half of the stock and on the more basal secondary branches of the primary branches of this region. In some stocks this basal portion is a tangle of stolons with few or no hydranths, the stolons arising in part in the positions of hydranth buds and in part as apparently "adventitious" outgrowths.

A little later it was observed, particularly in *Bougainvillea,* that stocks which were entirely without these stolons when first collected often developed them after a few days in standing water in the laboratory. This suggested the possibility that such transformation of hydranth buds into stolons might be the result of depression or inhibition and experimentation with low concentrations of various inhibiting agents and conditions, such as crowding in standing water, keeping in closed dishes, etc., was begun.

In these experiments the following chemical agents and concentrations[2] were used: KNC $m/10000$, $m/25000$, $m/50000$, ethyl urethane, $m/200$, $m/500$; MgSO$_4$, $m/400$, $m/1000$; LiCl,

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[1] I am indebted to Dr. C. C. Nutting for the identification of the *Obelia* and to Dr. Trevor Kinkaid for the identification of the *Bougainvillea* and *Gonothyræa.*

[2] Concentrations as given represent merely equivalents in sea water.

$m/50$; HCl, $m/1500$, $m/5000$; neutral red. With some concentration of each of these agents positive results were obtained, and crowding, keeping in closed dishes, and infrequent change of water also gave positive results.

In the experiments complexes, stocks or "colonies" lacking only the holdfast, or large complexes of stems and branches with a single cut surface at the basal end were used. These were placed in finger bowls holding about 400 c.c. of the experimental solution made up with well aërated sea water. When volatile agents were used the bowls were completely filled and covered with glass plates excluding all air, or all except a small bubble. Solutions were renewed daily or every two days, except in experiments to determine the effects of less frequent change. And finally, in some experiments the same concentration was continued throughout, in others the original concentration was replaced by a lower one, or the animals were returned to water after a day or two.

The figures are semi-diagrammatic but are all drawn from living specimens. Old stems, branches or thecæ which are empty because of disintegration or resorption of hydranths or retraction of cœnosarc are drawn in broken lines (Figs. 13–16, 18). Figs. 1–9, 11, 13 representing the development of stolons in earlier stages of experiment are drawn in outline without indication of cœnosarc, because all parts of stems and stolons contain it. In the other figures the cœnosarc is indicated by shading in order to show the later development and separation of stolons from the stock.

The chief purpose of the paper is the presentation of experimental data which show that transformation of stems and even of apical regions into stolons may occur under slightly inhibiting or depressing conditions. Questions of the range of effective concentrations, of regional, individual, specific and experimentally induced differences in susceptibility, and of the rate and degree of transformation are considered only incidentally or not at all.

TRANSFORMATION IN *Bougainvillea*.

Transformation of apical ends of branches into stolons often occurs in nature in *Bougainvillea*. In freshly collected stocks stolons are often found in place of some or all hydranths and

buds of the more basal regions, usually not more than the basal third or half, or as adventitious outgrowths from the branches of these regions. What factors are concerned in such cases is of course uncertain, but it may be pointed out that in these basal regions, which are often over-grown with plants and protozoa, less favorable conditions for respiratory exchange—retarded water movement, accumulation of CO_2, lack of oxygen—may directly inhibit and transform hydranth buds into stolons. On the other hand, if the whole stock is exposed to inhibitory conditions, the more basal regions, because of their lower rate of metabolism (Child, '19, '21; Hyman, '20), are in general less able to acclimate to such conditions than the more apical regions and may, therefore, undergo transformation, while other levels of the stock do not. Stocks which show no stolons at any level above the basal end when collected usually develop stolons over more or less of the stock after a day or two in standing water.

That such development of stolons from the more basal regions of stocks in water is not due to the presence or accumulation of "stolon-forming substances" in these regions or to any other specific factor is shown by the fact that this transformation can be induced in any or all regions of the stock, according to experimental conditions. The following experiments serve as examples:

1. Pieces of stocks in ethyl urethane, $m/200$. After forty-eight hours all hydranths disintegrated and numerous stolons developed, chiefly apical (Fig. 1) or subapical (Fig. 2).

2. Pieces in ethyl urethane for twenty-four hours, then returned to well aërated sea water. In ethyl urethane all original hydranths disintegrated and stolon development began. After twenty-four hours in water many subapical stolons were present, but new hydranths were developing and some stolons were transforming into hydranths and stems.

3. In ethyl urethane $m/500$ the original hydranths disintegrated and apical and subapical stolons developed within twenty-four hours, but after forty-eight hours many apical ends and in some cases the first subapical bud developed new hydranths and the stolons were inhibited (Fig. 3).

4. Pieces in ethyl urethane $m/500$ twenty-four hours, then returned to water, gave much the same results, except that in some cases the tips of the apical stolons themselves transformed
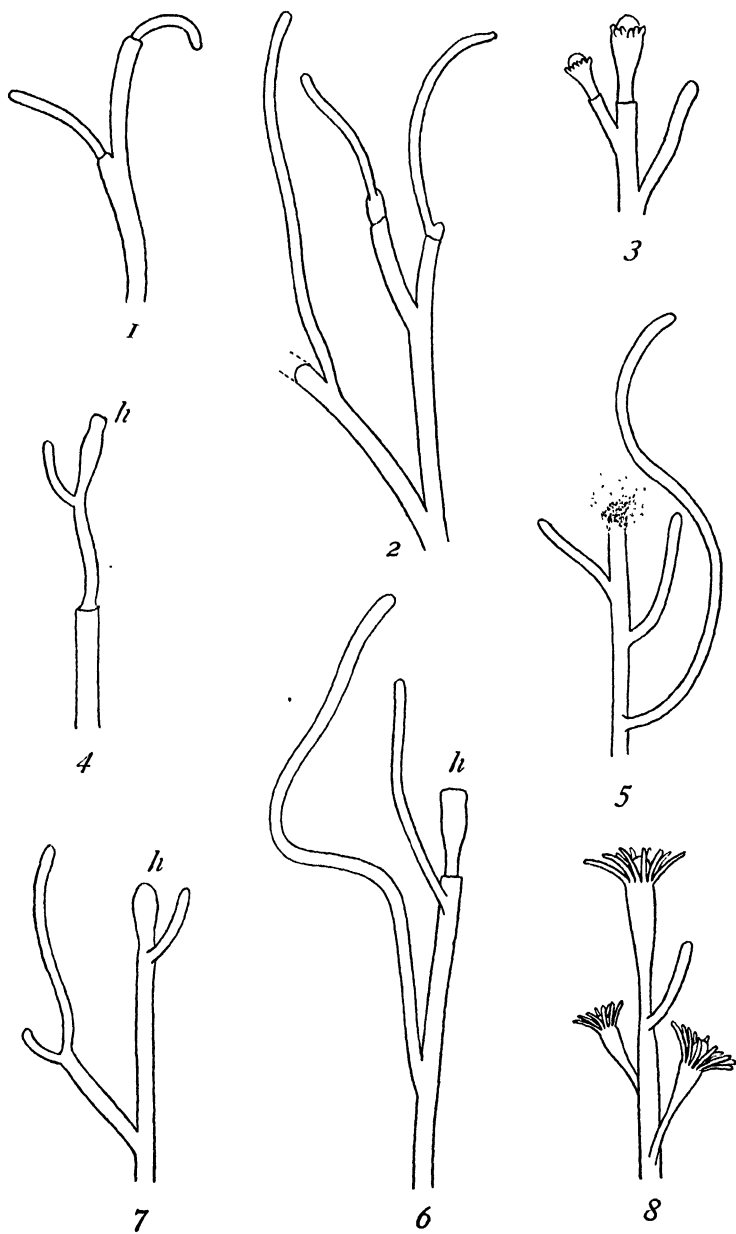
into hydranths after return to water (Fig. 4; *h*, hydranth bud).

5. In MgSO₄ *m*/400 almost all hydranths were disintegrated and subapical stolons grew rapidly within forty-eight hours (Fig. 5).

6. In MgSO₄ *m*/1000 disintegration of hydranths occurred and stolons developed much as in Expt. 5, but after forty-eight hours many new apical hydranths developed (Fig. 6; *h*, hydranth bud).

7. In sea water controls of Expts. 1–6 after forty-eight hours the original hydranths were more or less affected: tentacles were undergoing reduction or were completely gone and some hydranth bodies were disintegrated or disintegrating, or, in the case of the younger hydranths, resorbed. Subapical, or even apical stolons appeared on those axes in which the terminal hydranths were reduced or disintegrated · (Fig. 7) and rarely short stolons appeared on stems with persistent hydranths (Fig. 8). On some stems new hydranths were developing Fig. 7, *h*).

8. In KNC *m*/10000 hydranths became motionless within a few hours and in the course of three or four days disintegrated, but in the course of six days no stolons developed. Solution was then changed to *m*/20000 and left exposed to air to permit slow decrease in concentration. A few short, subapical stolons developed during the next eight days, but no development of new hydranths occurred and medusa buds underwent resorption. Evidently development and growth were almost completely inhibited by this concentration.

9. In KNC *m*/25000 older hydranths disintegrated and younger underwent resorption. Stolons developed in subapical and lower regions. After six days solution was changed to *m*/50000 and left exposed to air. During next eight days an extensive stolon system developed from nearly all apical ends of all stems and branches and also from basal cut ends of pieces. No hydranths were present but a few hydranth buds appeared on the stolons. Results essentially like those of following experiment.

10. In KNC *m*/50000 the original hydranths disintegrated and stolons appeared and after six days small, partially inhibited new hydranths were present (Fig. 9). Solution was then changed to *m*/100000 in open dishes to permit gradual decrease in concentration. During the next eight days the new hydranths were

resorbed and extensive stolon systems developed from practically all tips (Fig. 10). These stolons bear a few erect stems with hydranth buds (Fig. 10; *h, h*), but these did not develop further.
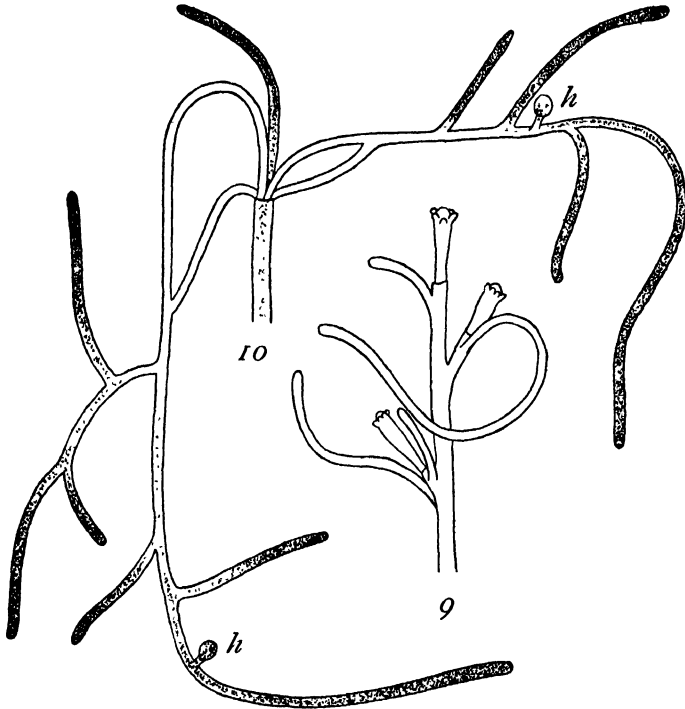
11. In HCl *m*/5000 ± pH 7.4, after forty-eight hours, most terminal hydranths of upper branches disintegrated and many subapical stolons present. Many hydranths at lower levels intact; no stolons. In controls in sea water, changed daily, terminal hydranths mostly disintegrated but new hydranths developing and very few stolons present.

12. In HCl *m*/1500 ± pH 6.9 hydranths disintegrate in one to two days, terminal hydranths usually first. After three days numerous stolons appeared in basal halves of pieces, but all development was inhibited in the apical halves. In sea water controls after three days the original hydranths were gone, many new hydranths were developing and few stolons appeared.

13. In pieces kept in standing water in closed vessels without air the changes during the first forty-eight hours are essentially

similar to those occurring in open dishes. More or less reduction and disintegration of hydranths and development of stolons occurs. Figure 11 shows the apical region of a stem in which the original terminal hydranth has reduced tentacles with disintegrating tips and the subapical hydranth bud has advanced but

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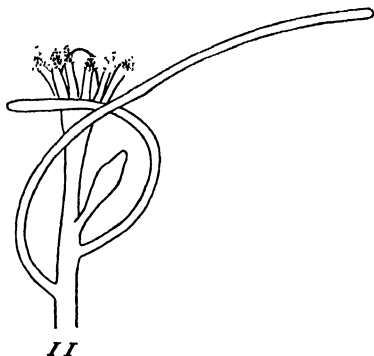
little during the forty-eight hours since collection. Below the bud two stolons have developed. In some other pieces in the same dish most or all hydranths appear intact, and no stolons or very few, chiefly in basal regions, are present. In still others apical hydranths are already completely disintegrated and stolons are growing rapidly.

In all cases in standing water the original hydranths disappear sooner or later and stolons develop, but a second and even a third generation of hydranths may arise. After a week or ten days, however, hydranths are usually entirely absent and all tips have either given rise to stolons or the cœnosarc is retracted. In short, the effect of standing water is essentiallly the same as that of the inhibiting agents, but less rapid. It is probably due in large part to accumulation of CO_2. There is always a decrease in pH in the dishes containing the pieces, often to 7.5 or 7.4 in the course of a week or less.

TRANSFORMATION IN *Gonothyræa*

Gonothyræa is much less susceptible to inhibiting agents and to standing water than *Bougainvillea*. The hydranths survive for several days or even a week under conditions which kill the hydranths of Bougainvillea in a day or two.

Some of the earlier experiments gave negative results as regards stolon formation, probably because the inhibiting conditions were not sufficient in degree or not continued long enough. Apparently however, the transformation occurs under less extreme conditions in stocks which have begun to produce gonozooids
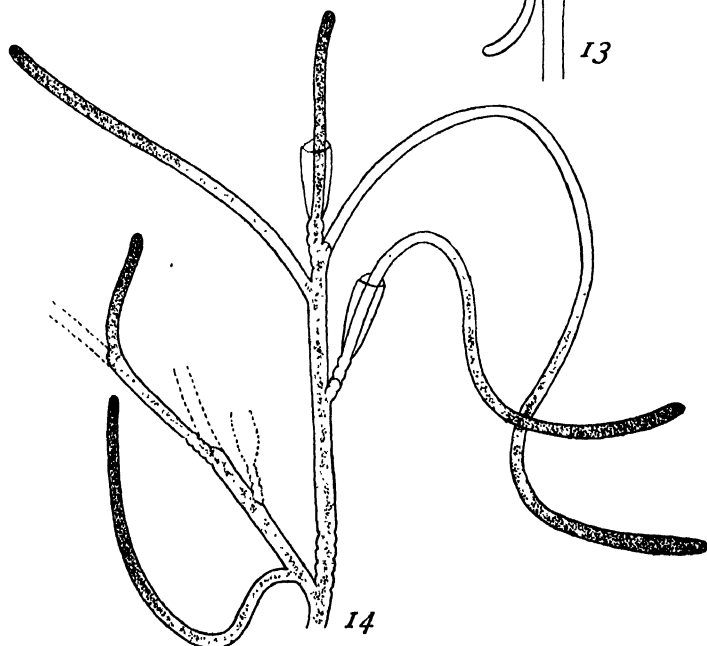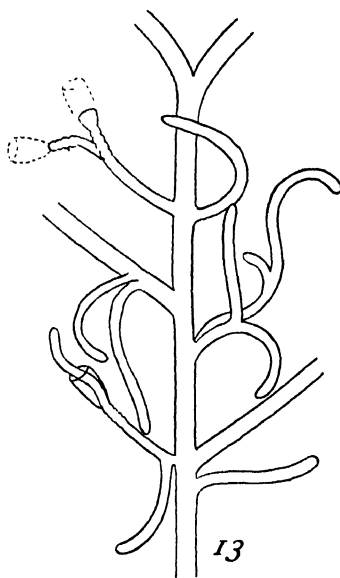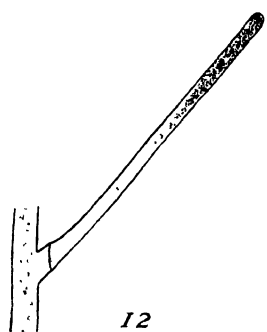
than in those which have none, but my data indicate that transformation will occur to some extent in any stock with sufficiently high concentrations of the agent used and sufficient time. Lack of material limited experiment with this species. A few examples are given.

Pieces in HCl $m/2500$ ± (pH 7.3) still possess some hydranths after four days. A few short outgrowths, apparently intermediate between stems and stolons have developed but no typical stolons. These pieces were then changed to HCl $m/1500$ ± pH 6.8 and after two days more showed numerous stolons in the more basal regions and outgrowths apparently intermediate between stolons and stems in the apical regions. These intermediate outgrowths are straight, support themselves free in the water and do not adhere to surfaces as do the stolons, but they show no annulation and they develop quite independently of hydranth buds (Fig. 12).

Fig. 13 shows the development of stolons in the basal region of a stock after a week in ethyl urethane $m/200$. In ethyl urethane $m/500$ transformation did not occur within a week. Figure 14 shows stolon development in the apical region of a stock after ten days in standing water. This stock bore numerous gonozooids in the basal regions.

TRANSFORMATION IN *Obelia*.

In *Obelia borealis* transformation was observed in HCl $m/1500$, LiCl $m/50$, chloretone $m/2000$, neutral red and also in standing water, but in water the stolons are less numerous than in the various solutions and after a few days new hydranths develop. In neutral red all parts stain opaque black, the hydranths disintegrate or are resorbed and stolons which are opaque black like other parts develop in large numbers. In this species stolons may be either terminal (Figs. 15, 16) or in the axils of branches (Fig. 17). Usually when they attain a certain length they separate from the parent stock, fall to the bottom and the tip continues to grow at the expense of lower levels until reduction to minute size and exhaustion occurs, or, if inhibiting conditions are removed, they may give rise to hydranth and stem.

Obelia geniculata behaves essentially like *O. borealis* as regards transformation and the stolons are of the same type in the two species. Another unidentified campanularian showed extensive

stolon formation after two days in standing water, many of the stolons being apical. Fig. 18 shows a case of apical transformation in this species.

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17   16   18
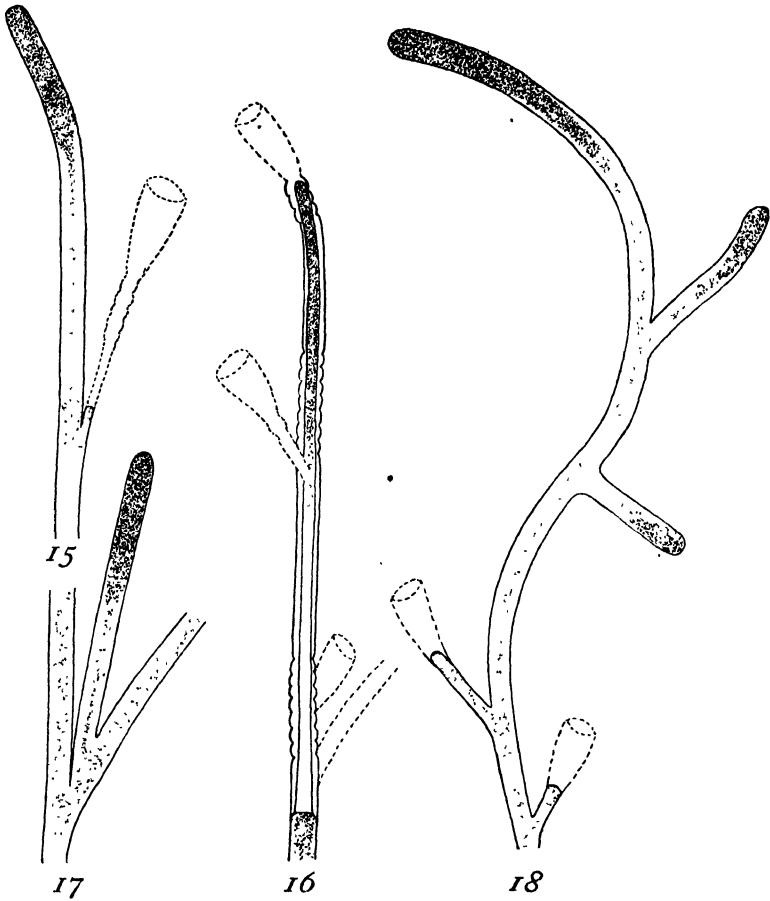
DIFFERENCES BETWEEN STOLONS AND STEMS.

There is usually no difficulty in distinguishing a stolon from a stem. The stem supports itself and is relatively rigid, the stolon is much less rigid and when it grows free in the water tends to hang downward as its length increases, until it comes into contact with a solid surface. Once in contact with a surface, the perisarc of the stolon adheres to it and its further growth is along the surface, but the stem does not attach itself to surfaces. It is not true, however, that contact is an essential factor in the origin

of stolons, but my observations suggest that in general stolons in contact grow to greater lengths and perhaps more rapidly than those free in the water. In the figures all the longer stolons are in contact with the glass of the container over most of their length (Figs. 2, 5, 10, 14, 18). The adventitious stolons in Figure 13 and the short stolons of Figures 1–5, 7, 8, etc., are not yet in contact with surfaces.

The stem grows only as part of a hydranth-stem complex or gradient, while the stolon may continue to elongate indefinitely without developing hydranths or hydranth buds. The direction of growth of stolons is apparently indefinite, or at least is readily altered, but the stem is usually straight. And finally, in those species in which annulations of the perisarc appear on the stems, the stolons are not annulated.

The "intermediate" outgrowths observed in *Gonothyrœa* in HCl (p. 190 and Fig. 12) resemble stems in their rigidity and definite direction of growth, but, except in the development of the first hydroid from the planula, stems do not grow out in this manner without at least a hydranth bud at the tip. If the difference between stolon and stem is primarily one of "steepness" or other difference of the gradient, such intermediate outgrowths are possible and there is no good reason for doubting that the outgrowths observed are really intermediate.

THE PROCESS OF TRANSFORMATION.

The first step in the process is the inhibition or depression of the hydranths. The changes in the hydranths differ according to species and degree of depression, and range from decrease or cessation of motor activity for a day or two with subsequent recovery to complete disintegration or resorption. In *Bougainvillea* all the original hydranths usually disappear, even in standing water, within twenty-four to thirty-six hours and new hydranths may begin to develop within forty-eight hours. In *Gonothyrœa*, on the other hand, the change from natural conditions to standing water has little effect on the hydranths; they remain intact for a week or more in most cases, but finally disintegrate or are resorbed as starvation advances.

The older, fully developed hydranths apparently always die and disintegrate, at least in large part, in all the species observed,

though it is possible that some portions may be resorbed, but younger developing hydranths or buds may be completely resorbed without visible loss of tissue.   In *Bougainvillea* even the younger medusa buds underwent complete resorption under the more extreme inhibiting conditions, *e.g.*, KNC $m/10000$ six days, then gradual decrease in concentration.   After two weeks the youngest medusa buds were completely resorbed, only the empty perisarc marking their position, somewhat older buds were partly resorbed and the most advanced buds had undergone disintegration.

The question of the nature of the process of resorption of hydranths was discussed some twenty years ago by Loeb ('oo) and Thacher ('03), Loeb postulating a liquefying enzyme and Thacher interpreting the process as a degeneration.   To all appearances the process is to some extent a real dedifferentiation.   There is no visible loss of tissue and from what we now know of metabolic relations in the hydroids a simple interpretation of resorption and retraction of parts seems possible.   The growing hydranths and medusa buds are regions of higher metabolic rate than the stems and are therefore able to appropriate the larger share of nutrition, but at the same time they are more susceptible to inhibiting or depressing conditions than stems (Child, '19, '21). When subjected to these conditions their metabolic rate decreases to a much greater degree than that of stems.   Under such conditions they may not only be unable to maintain themselves, but their tissues, instead of taking nutriment from other parts, may become, whether through autolysis or other factors, a source of food for other parts.   Consequently they may undergo decrease in size until nutritive equilibrium is established, or until the region with lower metabolic rate is more or less completely resorbed.   Whether individual cells actually die in such a process or merely undergo dedifferentiation is difficult to determine, but in the light of what we know of the possibilities of dedifferentiation in these animals, it seems probable that cell death does not necessarily occur.

In starving hydroid stocks in the laboratory the retraction of cœnosarc in some regions and its outgrowth in others has often been noted.   Tests of susceptibility and of permanganate reduction in such stocks indicate that the regions which are grow-

ing at any given time are regions of high rate of oxidation, while those which are undergoing retraction or resorption are regions of low rate. It seems probable then that in general the retraction of one part and the growth of another, particularly in starving stocks are associated with such differences in rate, the region of high rate maintaining itself and even growing at the expense of less active regions.

The normal relations and proportions of parts in nature must depend in large measure upon certain relations of rate of fundamental metabolism. When these metabolic relations are altered the form or proportions must change, and in simple, highly plastic forms such as hydroids, such changes may involve the complete resorption or atrophy of previously existing parts and the dominance and development of parts previously subordinate,

The stolon represents a physiological axis, a gradient (Child, '19, '21), but the data of susceptibility, $KMnO_4$ reduction and vital staining for some ten hydroid species examined indicate clearly that the stolon gradient is much less "steep" than the hydranth-stem gradient and that whenever, and wherever the gradient in a stolon becomes steep enough, transformation into a hydranth-stem complex occurs. In other words, the difference in rate between the hydranth bud or hydranth and the stem is greater than that between the stolon tip and the stem or the lower levels of the stolon.

According to this viewpoint, the stolon usually appears in nature as a basal structure, not because of the presence there of any "stolon-forming substances" but first, because this is the region of lowest metabolic or oxidative rate in the stock, and second, because new buds arising in this region are more or less inhibited by the dominance of the more active regions above. Probably the bioelectric currents resulting from the differences in electric potential between basal and higher levels are important factors in such inhibition. But whatever the factors involved, the partially inhibited axis develops in the form of a stolon. As I showed for *Tubularia* (Child, '15, pp. 91–2, 130–37), when the distance of the stolon tip from the hydranth becomes great enough, the stolon tip becomes physiologically isolated from the inhibiting action of the more active levels and transforms at once into hydranth and stem.

The experiments show, however, that stolon formation is not necessarily limited to basal regions of the stock, but may occur anywhere, even at apical ends, under inhibiting external conditions. In consequence of the differential susceptibility of different levels of the axis, the effect of such conditions is to decrease the steepness of the gradient. This change induces disintegration or resorption of hydranths, and new buds, instead of developing into hydranths, give rise to stolons. Moreover, a greater or less degree of physiological isolation of stem regions results from the disappearance of the dominant hydranths and in some species, as in many plants "adventitious" buds, i.e., buds not localized in conformity to the usual order, arise (Figs. 11, 13). But under the inhibiting conditions the buds develop as stolons, not as hydranths. Such adventitious stolons have been seen most frequently in the basal halves or thirds of *Bougainvillea* and *Gonothyrœa* stocks, but apparently may occur anywhere. And finally, the inhibiting conditions alter the steeper hydranth-stem gradients of terminal regions into the less steep stolon gradients and stolons therefore appear in place of hydranths.

Often more or less acclimation to the inhibiting conditions occurs in the course of a few days, and new hydranths begin to develop either from terminal regions which have not formed stolons (Fig. 7), from new buds, or by the transformation of stolon tips (Fig. 4). Such hydranth development retards or completely inhibits further growth of subterminal stolons near the hydranth, but the growth of terminal stolons may continue indefinitely (Figs. 10, 14, 15, 18) unless conditions are so altered as to induce their transformation into hydranth-stem gradients. In the case shown in Fig. 10, for example, each of the two chief stolon outgrowths gave rise at one point in its growth to a hydranth bud (*h, h*), but these two buds were unable to develop further than the stage shown in the figures and later underwent resorption.

SEPARATION OF STOLONS FROM THE STOCK.

In all species investigated continued growth of the stolon leads sooner or later to loss of continuity between its cœnosarc and that of the parent stock. In *Bougainvillea* (Fig. 10) and *Gonothyraea* (Fig. 14) such separation of stolon and stock usually

occurs gradually and only after the stolon attains considerable length, but in *Obelia* it usually occurs at a relatively early stage and the region of separation is more sharply localized (Figs. 15, 17). Such differences, however, are not entirely constant for the species, for *Bougainvillea* stolons sometimes show a definite level of separation and *Obelia* stolons sometimes do not.

After separation of the cœnosarc the stolon may remain connected with the stock by the perisarc. In the case of stolons hanging free in the water the empty perisarc usually breaks and the stolon falls to the bottom, attaches itself and continues to grow, the tip growing at the expense of more basal levels until exhaustion occurs, or until conditions are altered so that the tip can transform into a hydranth and stem. Such free stolons may cover many centimeters of distance, leaving behind them a tube of empty perisarc as they go, and decreasing in length as their substance is gradually used as nutrition. In the laboratory this growth may continue for three weeks or even more, according to temperature, and while transformation into hydranths often occurs in the early stages, it apparently does not take place, even in favorable environment, in the later stages, but the stolon continues to "creep" over the bottom until reduced to a minute amount of cellular material. And even when growth ceases the small masses of tissue in the perisarc remain alive for some time longer.

The separation and continued growth of these stolons receives a simple physiological interpretation in terms of the axial gradient. If the stolon is such a gradient, the levels of relatively high rate are able to live to some extent at the expense of lower levels. Under laboratory conditions, without intake of food, the growth of the stolon tip is possible only at the expense of other parts. In the early stages the stolon tip, as a region of higher metabolic rate than the old stem cœnosarc, is able to take material from the latter, but as the stolon elongates the growth of the tip occurs more and more exclusively at the expense of the lower stolon levels, because the stolon gradient, and consequently the nutritive concentration gradient, is limited in length and when the length of the stolon exceeds this limit, it can no longer draw on the stock for nutrition.

From this stage on, the lower levels of the stolon gradient

gradually lose material to the higher levels and finally the cœno-sarc of these levels disappears completely and separation of the stolon occurs. In Fig. 10 the two chief stolons have already sepa-rated from the original stock, and some of their longer branches are approaching separation from each other. In Fig. 14 also the two largest stolons are separated and a third is approaching separation. In Fig. 15 the terminal stolon of *Obelia* is almost separated, in Fig. 16 separation is complete, except as regards perisarc, and in Fig. 17 an earlier stage is shown.

After separation growth goes on as long as the regions of higher rate are able to take material from those of lower rate. In such stolons, even after a week or two of growth, the tip appears well fed and the cell layers are thick while toward the base the layers become progressively thinner and the cells more shrunken.

It is not yet known whether the rate of oxidation increases in advanced starvation in hydroids as it does in *Planaria* and various other animals, but apparently either this occurs at the lower levels more rapidly then at the upper levels of the separated stolon, or else the rate of oxidation in the upper levels decreases as the supply of nutritive material decreases. Either change leads gradually to the obliteration of the gradient, and as the cells become more and more alike in condition, growth becomes slower and slower and finally ceases.

According to this interpretation then the continued growth of such separated stolons in the absence of food from without is a simple physiological consequence of the fact that they represent physiological gradients and likewise the difference in appearance of the cœnosarc from the well filled tip to the shrunken, almost transparent base is another expression of the gradient. It may be suggested further that such stolons fail to develop hydranths in the more advanced stages of starvation because the gradient cannot attain the steepness necessary for hydranth formation. So far as I know, no other adequate physiological interpretation of these various facts has been advanced. The separation and continued growth of stolons may be an adaptation for purposes of reproduction under unfavorable conditions, but even if this is the case, the necessity for physiological interpretation still exists.

CONCLUSION AND SUMMARY.

It is evident that the formation of stolons in the hydroid species investigated is not dependent on region of stock, gravity, or contact, but rather on a certain degree of inhibition or depression, which may be determined either by relations to other parts of the stock or by external factors. Theories of physiological polarity based on distribution or direction of flow of hypothetical "formative stuffs" or upon molecular polarity and orientation afford no satisfactory interpretation of the facts presented in this paper. On the basis of such theories we must assume that placing the animals in standing water or in the experimental solutions must alter fundamentally the distribution or direction of flow of the formative stuffs, or must alter the molecular orientation or polarity in many different ways. But there is not the slightest reason for believing that such changes in conditions could accomplish any of these results. In terms of physiological gradients, however, all the facts are readily and simply accounted for and brought into line with other facts, and the earlier observations concerning such transformations and changes in polarity are likewise easily interpreted. In this field, as in many others the gradient conception affords a basis for the interpretation and synthesis of data which has previously been lacking.

In view of apparently persistent misunderstanding of the gradient conception, it is perhaps necessary to emphasize once more the fact that it is concerned with the physiology of development, not with heredity. In other words, the specific protoplasm of *Bougainvillea*, of *Gonothyræa*, or of *Obelia*, with all its hereditary potentialities, whatever these may be, is in each case the basis in which the gradient appears. This conception merely holds that, given this or any other specific protoplasm, the physiological gradient is an essential and fundamental factor in the realization of the hereditary potentialities in the form of an axiate individual of the species to which the protoplasm belongs.

The chief points are summarized as follows:

1. In various hydroid species the development of stolons can be induced by slightly inhibiting or depressing conditions, *e.g.*, low concentrations of ethyl urethane, $MgSO_4$, KNC, HCl, LiCl etc. and in most species even by change from natural conditions to standing water in the laboratory.

2. Stolon formation can be induced at all levels of the stock. The stolons may arise as adventitious outgrowths, by transformation of hydranth buds, or by transformation of terminal regions of stems after disintegration or resorption of hydranths.

3. These facts, together with data concerning the physiological gradients in hydroids indicate that the stolon axis is a somewhat inhibited gradient and less "steep" than the hydranth-stem gradient. The separation of stolons from the stock and the continued growth of stolon tips at the expense of lower levels in the absence of food are regarded as necessary consequences of the presence in the stolon of an axial gradient.

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THE DIGESTIVE SYSTEM OF THE PERIODICAL CICADA. II. PHYSIOLOGY OF THE ADULT INSECT.

CHARLES W. HARGITT.

The periodical cicada, now technically known as *Tibicen septendecim*, is well characterized by Marlatt (Bulletin No. 71, Bureau of Entomology, 1907, p. 11) as "undoubtedly the most anomalous and interesting of all the insects peculiar to the American continent. This cicada is especially remarkable in its adolescent period, the features of peculiar divergence from other insects being its long subterranean life of thirteen or seventeen years, during all of which time its existence is unsuspected and unindicated by any superficial sign, and the perfect regularity with which at the end of these periods every generation, though numbering millions of individuals, attains maturity at almost the same moment." Dealing with these several peculiarities and related problems of distribution, economic bearings, localized broods, etc., a large literature has accumulated since its first recorded advent at Plymouth, Mass., in 1623. But literature concerned with the more technical problems of its life history, for example, its embryology, morphology and physiology, is relatively small in volume and not of especially high value. Of some three hundred titles cited by Marlatt in the above-mentioned bulletin, by far the most of them relate to matters of habit, distribution, injuries caused, enemies, etc., and only an occasional reference to the anatomical or physiological problems concerned in its life. Much of this apparent indifference may be attributed to the highly obscure character of the life history of the insect, but not wholly so. For example, until the former paper by Hickernell appeared, there seems not to have been any critical account of the internal anatomy·of any of the several organ systems of the insect. Nor has particular attention been directed to what must be rather unique physiological processes in such an organism. It is the purpose in what follows to submit an outline at least of the chief physiological observations which

have been made, extending over a series of years, with an effort to correlate them with the clearly established anatomical and morphological features already described. In this connection will also be reviewed some physiological results of recent times which seem to have intimate bearings upon the immediate problems before us. While there has been intimate and continuous coöperation and mutual aid between the writers of this paper, in almost all phases of the research, it is frankly stated, however, that each is independently responsible for his own contribution.

The critical interest of the senior author in the morphology and physiology of this insect began over twenty-three years ago at which time its emergence in June, 1899, afforded a novelty in laboratory material which was presented before a large class in zoölogy. While in the external morphology of the insect there was ready recognition of the general equivalents, or homology, already familiar from laboratory studies of crickets and grasshoppers, it was quite otherwise when dissection was undertaken and a study of the internal anatomy was attempted. Here it was soon apparent that conditions were so different from anything before studied as to be puzzling in the extreme, and it was decided that this part of the subject was beyond profitable attempt at anything more than a general, and rather superficial survey, especially as it came in the hurried closing days of the college year. At a later time this problem was assigned to a graduate student, R. L. Henderson, reference to which was made by the junior author in his previous paper. The generally accepted view among entomologists was that this insect seldom or never feeds during adult life, and in part this led likewise to the view that the digestive organs were more or less degenerate or even atrophied. Such was my own conclusion from the preliminary study above referred to, and presented in a paper read before the County Academy of Science in whose proceedings the latter appeared in print. When Henderson undertook the work assigned to him just cited above, he was early forced to discredit my earlier conclusions on this point; and while unfortunately he did not live to complete his research, some of that which he left in manuscript shows that he had obtained clear evidence of

the continuity and functional activity of the entire digestive system. The recent work by Hickernell has corrected and extended the investigation in a thorough and convincing way.

FEEDING HABITS.

A consideration of the feeding habits of cicadas is important in the present connection, as conditions in the digestive organs will be influenced by the reaction of the insect to food. As pointed out in the previous article, it has been maintained by various students of these insects that they seldom if ever take food during the adult life. Later studies by Quaintance, to which Marlatt has yielded a measure of assent, and the researches of the former paper already referred to, clearly establishing the functional efficiency of the digestive apparatus, renders the conclusion inevitable that there is no intrinsic difficulty in the views of these observers as to the feeding habits of the insect during the adult period of its life. The senior author has studied the food habits of this insect at several intervals for some twenty years and is not convinced that the accounts by Quaintance just mentioned are fully confirmed. Very special attention was given to this point during the appearance of the brood in this locality in the year 1916. In the main, it confirmed previous studies in 1899. In both cases attention was directed to two phases of the adult life, namely, that immediately following the emergence from the nymphal stage and during the early free-living condition, when it was believed the occasion to replace the wastes of this ordeal might express itself in free food taking. Furthermore, it was during this period of early adult life that accurate observations could be most easily made. But repeated observations at this time failed to show a single decisive case, though at times the attitude of the insect was such that it was necessary to disturb it in order to make certain that it was not actually feeding. Another period for observation was that about a week or ten days later when activity was very marked, especially among the males, in view of the growth of the reproductive organs and cells at this time. It was assumed that here was a special·stage when need of food must be rather urgent. But here again, very few cases even under cage environment were distinguished. It must be admitted that during this period few specimens could

be found in which these observations were easily studied and, as before stated, it was found very difficult to distinguish between a merely resting or quiescent state and that of the attitude of the insect in feeding without disturbing its pose. To make this a matter of precision, numbers of insects were placed in breeding cages in the laboratory. These cages were provided with fresh stems of shrubs, and twigs from trees, and thus kept renewed daily for some time. At the suggestion of the senior author, an out-door insectary was constructed upon the lawn by using mosquito netting which was spread over growing shrubs and carefully fastened to the ground. In this enclosure, scores of specimens were kept under close observation for many days. But while cases of apparent feeding were now and then observed among these specimens, upon closer examination it was found that actually a very few were feeding, comprising less than 1 per cent of those under these conditions of observation. In view of these results, it has seemed to us that one could hardly accept the contention of Quaintance already cited that there was "frequent and general" feeding during the insect's life. The senior author's field observations on this matter have been rather extended. He has never observed what has been described as the abundant "exudation of greater or less quantity of sap from the puncture" of such feeding specimens, or "the trunk and larger limbs quite wet with the sap which had escaped in this way," as described by Quaintance. These out-door studies were often made in the early hours of the morning when dew was dripping from every bush but it was not supposed that this was in any case an exuded sap of the plant tissues upon which the insects were found! But neither under these field ·observations nor in those carefully restricted to the inclosed insectaries already referred to, was the writer able, except in the rarest instances, to convince himself that feeding occurs at all; certainly it is not a *common and general feature of adult life*. Furthermore, numerous dissections made of the insects have failed to show the gorged conditions of stomach and rectum which is described by several as plethoric to the point of rupture on the slightest disturbance!

Feeding habits among other animals of similar life cycles confirms what has just been pointed out. It is well known that many other insects have similarly anomalous habits and life

histories, and that among them are various species of ephemerids. From the early accounts of these insects so graphically described by Swammerdam ("Natural History of Insects," Eng. trans., 1758, pp. 103–27), on to the present time, it has been common knowledge that these and other species live as larvæ for many months or even two or three years an aquatic life. During this time, they are voracious feeders. Finally, at the time of metamorphosis, they emerge in enormous swarms during the summer, chiefly at evening, having relatively few hours of adult life, during which mating takes place and soon after the discharge of eggs, the early death of the adults. During this brief period of adult life, they take no food; the digestive system, and especially the mouth organs, being so imperfect as to render them incapable of active function. But like the cicada, these insects have the body tissue loaded abundantly with fat, which, in view of the extremely brief period of activity, can hardly be needed for nutritive purposes, but is doubtless utilized in the main for the rapid growth and perfecting of the generative organs and their products. I have verified these observations repeatedly and I am quite able to confirm what is more formally stated by Metchnikoff ("The Nature of Man," pp. 271–277). He shows that the rapid death following the act of mating and the discharge of eggs cannot be attributable to this act in itself since many males which have not undergone this action owing to the great excess of male insects yet die as promptly as do others which have participated in the process. He also shows that death cannot be due to the presence of pathogenic organisms since diligent search has failed to reveal their presence; and further that it is not due to phagocytic action, since the organs show no indication whatever of such invasion. He suggests the probability that such rapid death may be the effect of the early death of the cells of the nervous system, yet gives no evidence in support of the suggestion. In a later work, "The Prolongation of Life," this author emphasizes the significance of natural death in many groups of lower animals and the unique modes of providing against its hazards to the continuity of the species. Among these, he cites observations and experiments upon Rotifera (pp. 113, 118). "The whole course of life from the laying of the eggs until death lasts only about three days and is probably the shortest duration

of life in the animal kingdom. . . . The little males begin to swim soon after hatching, the wheel apparatus and the musculature being vigorous. They seek out the females, as their reproductive organs are mature at the moment of hatching. The transparent body, which is devoid of digestive apparatus, swarms with mobile spermatozoa. As soon as the male has seized the female, he discharges the contents of his body. It might be supposed that such an evacuation would cause violent perturbation of the system leading to the death of the organism. But the males are able to live for many hours after having accomplished their function, and the period represents a third of their natural duration of life. Moreover, I have isolated males from the females without any prolongation of their life. In one experiment, I isolated two males and placed a third in company with two females. It was the third specimen that lived longest. There can be no doubt but that the death of these male rotifers is natural in the fullest sense. The females, although they are provided with complete digestive organs, do not escape a similar fate. In some Ephemeridæ, which supply good cases of natural death, the end comes after a few hours of adult life without any sign of degeneration of the organs. As in others (Chlöe) life lasts several days without food ever being taken, it is clear that inanition is not the cause of the swift arrival of death in the first set."

As will be perceived, these citations from Metchnikoff relate primarily to distinctly different problems. But they are not without a measure of significance in connection with those under review. One point of importance is the fact that in these widely differing groups of organisms certain very fundamental functions, especially that of reproduction, take place normally during a period of inanition. Granting that the phenomena related of the Rotifers may be somewhat exceptional, and of only incidental significance, certainly those exhibited by the Ephemerids are clearly significant and pertinent, and have much in common with those so conspicuous in the life history of *Cicada;* thus accentuating the occurrence of kindred phenomena in widely differing organisms.

FAT STORAGE IN ANIMAL ECONOMY.

The phenomenon of storage of fat among animals is a fact very well, and long known, and its physiological significance has been also generally recognized. Its occurrence in animals which pass long periods of hibernation, during which no food is taken, hence are dependent upon those reserve sources for sustenance, is a matter equally well known and common in many groups of animals. Among these are mammals, reptiles, amphibia and fishes. In the last group are cases in which such reserves are accumulated to meet extra and unusual demands which are involved in extended migrations to distant spawning grounds. And further dependence upon this store of energy is required for the maturation and fertilization processes involved in the reproductive crises common to many of this class of animals. This phenomenon is known in numerous species among which the salmon is a familiar example, with the rather tragic consequence that this climactic performance is usually followed by immediate or early death of the organisms.

Some recent investigations and experiments of rather striking importance have been made by Prof. C. W. Greene concerning the physiological processes, both of the storage of this reserve material and its later resorption by the tissues. (Bull. U. S. Bureau of Fisheries, 1914, pp. 73–138.) Professor Greene has studied this especially in the King salmon during the long fast of the spawning migration. He shows how the storage takes place in the musculature and connective tissues during the late growth, and especially the voracious feeding just prior to the migratory ordeal which involves hundreds of miles up great rivers and against many and serious obstructions. The energy consumed during this ordeal must be supplied by these reserve sources of nutrition. And as just pointed out, the additional demand involved the growth and ripening of the sexual products and actual spawning of these at the end. It will be at once perceived that for such an ordeal very large resources of energy must be available and these are to be found almost wholly in these reserve fat materials. Many other such experiments have been made upon various species of animals such as amphibia, reptiles, etc., all going to sustain the above cited findings;

namely, that the storage of potential energy in the form of fats or surplus proteids is an obvious provision for maintenance of vital functions during periods of reduced or suspended activity, but which is made available by a reversed metabolism, brought about by the operation of identical or similar enzymes, as shown below. Greene has, by actual experiments and extended observations during these migrations of the salmon, shown with clearness and convincing results the entire physiological history of the absorption and storage of fats, and its later transportation to the various tissues and organs concerned. He also critically reviews the earlier work of Miescher along these lines and emphasizes its values, at the same time showing certain of its defects, especially its erroneous contention that the fats found in muscular tissues were degeneration products; and shows convincingly that the presence of fat in such tissues is a result of infiltration and "that intracellular fat of the King salmon is an expression of the nutritive state of the muscle. It is a loading of fat by a process of infiltration and is not a degeneration of the muscle substance." He next points out the applicability of the same discovery by Kastle and Lovenhart of the reversible action of lipase and, as a consequence, gets an insight into the mode of transportation of fats from tissues to tissues in the animal body. (*Ibid.*, pp. 123–125.) These researches of Greene throw fresh light upon very similar problems of reserve energy of storage fats well known in invertebrates. For example, the cases of the Ephemerids, Lepidoptera, and the periodical cicada, all of which show points of close similarity to the foregoing. Among insects, this reserve material is accumulated chiefly in a peculiar organ known as the "fat body" which is "of various shapes," according to Packard, "more or less lobulated and net-like and covers parts of the viscera, also forming a layer under the integumen. The tracheal endings are usually enveloped by the fat body. It is larger in the larvae than in the adults, especially in Lepidoptera, in them forming a reserve nutrition used during the metamorphosis and during the formation and ripening of the eggs and male cells." (p. 419.) According to Wheeler whom Packard quotes, this fat body is of mesodermal origin, and differentiated from portions of the cœlomic walls, hence of metameric origin. Numerous more or less conflicting accounts have been given as to the partic-

ular function of this body. For example, Marshal regarded it as a urinary organ; Graber regarded the entire system of the fat bodies as a simple many-lobed lung; a view likewise taken by Landois; but Schäffer took the view, now generally held, that it is a reservoir of nutrition from which the organism may draw during times of special stress or emergency. The case of the cicada is peculiar; for its whole larval and pupal existence comprises from thirteen to seventeen years of underground life devoted especially to feeding and growth. These finally culminate in the crisis of reproduction which lasts only two or three brief weeks. But during these weeks, feeding is almost wholly lacking as has already been previously shown. When first emerging into adulthood the body of this insect is literally gorged with storage fat and related reserves. But these rapidly decrease with growth and development of the reproductive cells, and with the maturation and discharge of these cells, this reserve supply becomes rapidly exhausted, especially in the male, and the female completes its exhaustion in the arduous task involved in puncturing branches and twigs for receptacles in which the eggs are laboriously deposited. These functions completed, the vigor of the insects rapidly declines, since the storage being exhausted and taking no new supplies, they rapidly decline and die. As an interesting incident bearing upon the matter, may be stated the fact that in the use of these insects as an article of food, which is common among American Indians, they are taken exclusively at the time the insect emerges in its mature form, or at least, very soon after, for at this time this storage matter is at its best and later, of course, rapidly deteriorates. At this period, also, the insects are preyed upon by hogs, fowls, and such birds as feed upon them, since only at this time are the insects easily available. For birds, they can be taken during the entire life period of a few weeks, but naturally, are most sought in early life when they are more easily captured.

There are many analogous features between the physiology of fat storage as shown in the foregoing citations from Greene's experiments and what probably takes place during the life cycle of these insects, some before mentioned. Among others the following are of interest.

1. The relatively long and probably more or less continuous

periods of feeding and growth. Experiments show that for the salmon it may be five to eight years; for the ephemerids two or three years; while for the cicada thirteen and seventeen years. In these groups this long period is now believed to be generally concerned in accumulating reserve potential energy, most of which will sustain an important relation to the brief, but crucial period of activity and perfecting the reproductive elements, and their union for the preservation of the species.

2. In each of these groups this actively cumulative growth and storage of energy, followed by a relatively brief period of reproductivity, gives that anomalous reaction of decline and death a unique significance.

3. Corresponding to these extended and painstaking researches of Greene I know of nothing among insects or other invertebrates; but the remarkably analogous aspects of the cases lead me to conclude that the physiologic activities involved in the latter are more or less similar; in some respects identical, with the former.

THE POSTERIOR CROP.

Under this caption, the junior author in his earlier paper has described a most unique and anomalous organ, clearly, as I believe, a part of the digestive system. For a full account of its anatomy reference may be made to his description in the paper just cited. It must suffice here to briefly summarize its main features. During nymphal life, as is well described in the second section of this contribution, it is rather small, "with walls of uniform texture and much folded. But in the adult, the walls of the organ are distinctly variable in thickness. The outer surface of the organ is closely apposed on all sides by fat. This probably has something to do with the collapsed condition of the tube in this region." A recent popular paper by Snodgrass descriptive of this insect designates this organ on the contrary as a part of the respiratory system. But, as will be shown later, this seems decidedly erroneous. In my earlier account (Proc. Onon. Acad. Sci., 1903, p. 51) I conceived it in adult life to act in some way as an organ for aiding in the absorption of fat, its epithelium in many cases being more or less charged with globules of fat. This was confirmed by the work of Henderson who also

showed that in no case of his numerous dissections, more than two hundred in all, of either nymph or adults, did he find traces in this organ of undigested food.

All this is abundantly confirmed by my own later work as it in turn also confirms earlier observations as to the feeding habits of these insects. Likewise, this is found borne out by the microscopic sections of the canal through every region of the mature insect by the junior author as shown in his previous paper. But there are certain rather puzzling features in this particular organ in adult life. It does not appear clear that its increase in size at this time can be due to a reservoir function, unless feeding be increasingly active during later life, which certainly does not seem to be the case. Again such a view seems to be in direct conflict with the histological character of the organ which shows clear evidence of degenerative changes in its lining epithelium. On the other hand it seems to conform with the view just previously expressed that it is during this stage apparently functionless in any active way, and that as the storage elements are resorbed the organ reacts in consequence, its walls expand to occupy the visceral spaces which earlier were filled by storage matter and reproductive organs, which in turn accounts for the attenuated condition of the epithelium described by Hickernell 'as above stated.

A further fact in this connection remains to be noted, namely, that these adult insects leave no signs of excretory wastes, such as defecative products. The writer has handled living specimens by hundreds, taken at various times, some kept under bell jars, others in clean breeding cages, as well as others still which were freely handled, but has not seen at any time evidence of defecation. Of course, the liquid sap upon which these insects feed might, and doubtless does show less of solid waste to be discharged, but certainly there are unused elements even•in such foods as sap which doubtless are extruded; as are those heavier products whose wastes are so conspicuous in the life surroundings of most feeding insects. The well-defined and thick-walled rectum of the cicada goes to support this view. This point is made as a matter of fact which should not be overlooked; but so far as I am aware it has not been given attention heretofore. Were feeding at all frequent or general in the adults such solid

excreta could hardly fail of notice. Their absence, therefore, can hardly be other than highly significant of the lack of active digestive operations during adult life, and is in entire accord with the fact of the entire absence of alimentary elements in the tract as above cited.

During the progress of this work my attention was called to a popular paper by R. E. Snodgrass on the Seventeen Year Locust (Smiths. Rept. for 1919, Washington, 1921, pp. 381–409), in which there appear certain views rather sharply in conflict with those herein maintained, and which call for some brief attention. Its anatomical points are reviewed by the junior author in the section which follows. But it falls to me to notice phases of feeding habits and others of a physiologic nature. In reply to certain inquiries submitted to Snodgrass he was kind enough to write me quite freely as to the questions, and also sent a specimen of transected insect to show the highly cavernous aspects of late life, and to afford what was suggested as a demonstration of the tracheal nature of the so-called "air-chamber." This, I examined with care, but cannot accept as demonstrative, since there were no distinct evidences of its tracheal structure, as the junior author conclusively shows in the histologic demonstrations of all phases of the typical and deteriorative epithelial cells occurring, and an entire absence of chitinous elements or tænidia in the organ.

Of this "airchamber" Snodgrass states that it "receives its supply of air directly through the spiracles of the first abdominal segment." If this were so there should be unmistakable evidence of its being a paired organ, as in the bee; but of this there is no evidence whatsoever, a fact which he admits but claims such to be the case in the dog-day cicada. This I have not been able to discover from actual dissections, or from serial sections of the regions; as shown by Hickernell in the earlier paper.

Referring to its function as related to the respiratory system Snodgrass without hesitation discredits the view of Graber, that it may have some relation to the tympanal organs, but says, "We shall probably have to fall back on the old prosaic explanation that bulk of body is maintained with corresponding weight eliminated—a combination specially favorable to aërial life." But as Packard long ago pointed out (cf. p. 457), this assumption

is erroneous, "The body of the insect during flight not being lightened by the air in the sacs." Submitting this point to two of my colleagues of the department of physics, Doctors Porter and Packard, I am assured that it is entirely correct.

It seems rather certain, therefore, that neither from its struc-ture, nor yet from the "old prosaic explanation" of an adaptation to aërial life, does he sustain his conclusions. Granting the ramifications of tracheæ over the organ no more makes it respira-tory than does a similar disposition of tracheæ over the viscera or the musculature constitute them such. Apart from the names by which Graber designates the organ, namely, "Tracheenbläse" and "Luftsäcke," there is nothing in his account which supports the views of Snodgrass. Graber's problem was the "Tympanal-organe," not respiration. I believe therefore that Snodgrass has misconstrued Graber's work so far as it is applicable to the matter at issue, and that his view may be dismissed as devoid of structural or functional evidence as a respiratory organ, or as an accessory "Tympanal organ," as claimed by Graber (p. 282–3).

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THE DIGESTIVE SYSTEM OF THE PERIODICAL CICADA, TIBICEN SEPTENDECIM LINN. III. MORPHOLOGY OF THE SYSTEM IN THE NYMPH.

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In an earlier paper, the form and relationships of the various parts of the digestive system of the adult cicada were considered. It was found that in both sexes the system is complete and well organized but much complicated by a winding and twisting together of its parts, so that the continuity of the system could be established only with some difficulty. The intertwining intestine, esophagus, and malphigian tubules in the anterior part of the body cavity makes the "internal gland" or filter which seems to be characteristic of so many of the Homoptera. This structure, while different in some respects in the cicada from that described in other homopterous forms, has a general arrangement which is similar.

It was found further that certain organs of the digestive system change shape markedly, as the insect increases in age, but that the ground plan of the alimentary tract remains the same throughout the life of the insect and that the system does not degenerate or become broken as has been believed by certain workers.

Because of the numerous peculiarities which were found in the adult digestive organs, it seemed worth while to examine the nymphal stages with the object of determining what conditions exist there and also to determine, if possible, the origin of some of the peculiar structural relationships. For, although the peculiarities of the homopteran digestive tube have been recognized for some time, the beginnings of these peculiarities in embryological history have not been figured or explained in many cases. It can at least be established whether the peculiar windings of the digestive organs arise at the time of one of the numerous moults or are to be traced back to the egg itself.

The consideration of the morphology of the digestive organs of the Homoptera dates back to the work of Lubbock, Leydig,

and Ramdohr about the middle of the last century. Lubbock described the digestive organs in *Coccus hesperidum*. He observed the complicated windings in the anterior region and called this complex the internal gland.

Dufour (1833) described the digestive apparatus of certain Hemiptera but the significance of some of the structures which he described apparently escaped him. In fact, it has been only recently that the digestive systems of any great number of Hemiptera have been worked upon and the true nature of the structural peculiarities of the organs determined.

Witlaczil ('85) studied the anatomy of the Psyllidæ. In *Psyllopsos fraxinicola* the digestive canal has many of the peculiarities which have been noted in the cicada.

Berlese (1909) describes the digestive organs of certain scale insects. In these insects the rectum is large and extends anteriorly as far as the esophagus. This results in a knitting together of rectum and esophagus and also causes the intestine to describe a complete circuit of the abdominal cavity before it finally joins the rectum. This condition, while resembling it superficially, is entirely different from the arrangement found in the adult cicada since in the latter the enormous enlargement affects the mid-gut while the rectum is relatively small.

Licent (1911) gives an account of the digestive organs in the Cercopidæ. In this family there is also a loop made by the mid-gut which curves backward so as to become intimately associated with the anterior part of the canal. Licent believes with Berlese that this complication acts as a filter, allowing the watery part of the food to diffuse directly through the walls of the fore-gut into the cavity of the hind-gut and in this way saves the mid-gut for digestive activity upon the more concentrated food mass. The nutritive substances, greatly diluted with sap, are thus concentrated and, for the most part, digested in the mid-gut.

Kershaw (1913) recorded his observations on the digestive organs in *Siphanta acuta*, a flatid. This form has a large reservoir or crop, which from its junction with the esophagus just within the abdomen, extends anteriorly above the esophagus through the thorax and practically fills the epicranium above the brain. It also extends posteriorly beneath the heart and above the rest of the internal organs, almost to the tip of the abdomen. It is

interesting to note that this food reservoir is similar in most respects to the enlargement in the digestive tube of the cicada, and furthermore that it attains its enormous dimensions only in the later adult life of the insect. In this last respect it further resembles the condition in the cicada.

From the above accounts taken with other works not quoted here, it seems evident that the digestive systems of the related forms of the group under consideration studied to date have, in general, the same ground plan and that their specializations although varying in degree, are mostly of the same kind. These structural complications have led to different interpretations of the relationships and functions of the various organs, but it seems likely that there is a much closer similarity in these respects than at first appeared to be the case.

The conclusions here presented are based upon a study of nymphs of *Tibicen septendecim* Linn. of four different sizes, viz. 2½ mm.; 7 1m.; 14 mm.; and 21–24 mm. This series covers practically the entire period during which the insect lives under ground. The 2½ mm. nymphs are only recently hatched from the egg, while the 21–24 mm. individuals were full grown nymphs dug from the ground about one month previous to the general emergence and transformation of the brood. According to Marlatt, the nymphs of 7 mm. body length are about four years old, while the 14 mm. ones are about ten years of age. These relationships between size and age were established by workers in the Bureau of Entomology, who followed a complete life-cycle of one of the cicada broods.

The method of serial sectioning and reconstruction was followed throughout this study. The impossibility of tracing the alimentary canal by means of gross dissections is even more apparent in the immature forms than it is the adult.

In describing the digestive organs as found in cicada nymphs of different ages, it would be more logical to begin with the youngest and follow the developemental series until the adult condition is reached. However, in an earlier paper the adult digestive organs were described and the descriptions and comparisons here given have been written keeping the adult structure in mind. Obviously, in such a situation it will be easier for the writer and more understandable for the reader to work back-

wards through the series of nymphal stages where structural variations are relatively slight, as between two successive stages, than to jump from the adult condition back to the earliest nymph and then work up to the adult condition once more. It is believed that a description of these different stages in reverse order is warranted in the present instance in view of the circumstances outlined above.

The alimentary tract in nymphs of 21–24 mm. length has the same general arrangement of parts as is found in the adult. The form and structure of the individual organs varies, however, in some respects. The posterior crop (Fig. 1, *pc*) always has its walls wrinkled and contains a tortuous lumen, as if the structure had collapsed completely, while in the adult this division possesses smooth, thin walls surrounding an enormous lumen. The size relations of some of the other parts also vary as is shown hereafter. A general view of the entire system as seen from the left side is represented in Fig. 1. The esophagus is a simple tube, uniform in diameter, and leads into the anterior crop (*ac*). The latter with its dorsal adherent "internal gland," is of about the same relative size as it is in the adult. The anterior crop empties through a narrow opening into the posterior crop (*pc*). The latter is greatly unlike the corresponding division in the adult. Its relative length is much the same but its walls are uniform in texture and much folded throughout. In the adult the walls of the organ are distinctly variable in thickness and are not folded to any extent except at the extreme anterior end where the cæcal projection runs ventral to the anterior crop. The outer surface of the posterior crop is closely apposed on all sides by fat. This probably has something to do with the collapsed condition of the tube in this region. At any rate, its opposing walls almost touch each other throughout its whole extent, thereby making the lumen narrow and irregular (Fig. 4, *pc*).

The ascending intestine is relatively larger in diameter in the 21–24 mm. nymph than it is in the adult. Its general course and connections are the same as in the adult but its size, both externally and with respect to its lumen is noticeably greater (Figs. 1 and 4, *at*). The ascending intestine enters the internal gland in the same fashion as it does in the adult. The descending

intestine also emerges from the complex of tubes as it does in the fully developed insect.

The descending intestine is relatively smaller in this stage than it is in the adult condition. Emerging from the internal gland it runs in a general posterior direction as in the adult, finally making a knot or coil (Fig. 1, *kk*) just before emptying into the rectum. There are some variations in the histological structure of the epithelium in its walls but the tube is easily recognized in section when one is familiar with its microscopic structure in the adult.

The rectum does not differ greatly from that in the transformed insect. It receives the descending intestine and then gradually narrows until the anal opening is reached.

Among the younger nymphs there are variations in size and arrangement of the digestive organs but these are slight as compared with the structural differences shown between the organs of the early nymphs and those about to transform to the adult condition. In nymphs of 14 mm. and 7 mm. body-length there is not enough variation in the arrangement of the organs in the two stages to warrant separate description. Figure 2 is based upon specimens of 14 mm. length but, except for size, the same figure applies to the shorter and younger stage. Esophagus, anterior crop, and internal gland are practically the same as in the stage previously described. The remaining portions of the system, however, differ greatly in many respects.

The posterior crop does not have any suggestion of saccular structure in these early stages. Its walls are folded as in the other previously described specimens but it does not assume the enormous diameter common to that division in the older nymph and adult. In fact, the ascending intestine has a larger diameter in this stage than does the posterior crop. Its course is almost straight through the center of the body until it reaches a point just anterior to the rectum. Here it joins the ascending intestine.

The ascending intestine (Fig. 2, *at*) is exceedingly prominent at this time. It is much convoluted and extends posteriorly in the region of the rectum, from which place it runs anteriorly. For the most part it runs along the ventral surface of the body and when it reaches the internal gland it disappears as in the adult. The interweaving of the posterior crop and the two intestinal divisions seems difficult of interpretation when first

studied in sections. However, the general ground plan is soon seen to be in no way different from that in the stages previously described.

The internal gland has the same structure here as it does in the older nymph. In size, it is of course, smaller but sections show identical parts in the two stages.

The descending intestine and rectum have the same form and arrangement in the 7 mm. and 14 mm. nymphs as they do in the later stages. The diameter of the descending intestine is very small. It runs close to the dorsal integument in some places and might easily be missed in studying sections. The characteristic coil (Fig. 2, *kk*) exhibits a convolution which is practically the same as that described for the later stage.

Examination of Figs. 1 and 2 makes it clear that there is no great difference in the arrangement of the digestive organs of the nymphal stages considered. It is also true that in the $2\frac{1}{2}$ mm. nymph there is not enough difference in structure or arrangement to warrant making a separate figure to represent conditions there. This means that the plan of the digestive apparatus is not altered practically throughout the entire underground existence of the insect. The variation in the size of certain organs at different periods in the life-history suggests either that the .nymph does not feed continuously or that there is a change in the function of some of the organs as time goes on. The former supposition is probably correct for it is known that these immature forms have alternate periods of feeding and resting.

The ascending intestine is found in some sections to have its epithelial lining made up of enormous cells filled with granules. In other cases we have the condition represented in Fig. 4, *at*, where the walls of this organ are thin and attenuated. These variations probably represent different phases of functional activity and are not to be interpreted, therefore, as indicating any change in the plan of digestive activity.

The complication of organs in the anterior region which has been called the internal gland, arises at a time earlier than that represented in the stages here described. Fig. 3, which is a transverse section through the internal gland region of a 24 mm. nymph, shows all the parts arranged in a manner similar to that in the adult. In Fig. 5, which is a like section through the same

region in the 14 mm. nymph, the same organs are found as before, but there is slightly less complication in the way of folding and intertwining than is found in the later stages. Figures 7 and 8 represent transverse sections through the anterior region of the digestive organs of a 2½ mm. nymph. Here again the various organs are seen to have assumed a position similar to that in which they are found in the later nymphs and adult. The peculiar relation of anterior crop, intestine, and malphigian tubules is, therefore, established in all these nymph stages the same as in the adult. In seeking the origin and significance of this arrangement, it is necessary, then, to go back to the development of the embryo within the egg.

In Figs. 1 and 2 the malphigian tubules have not been represented. They are present in the same number and arrangement as in the adult. They have been left out of the above figures since they only tend to obscure the clear representation of the digestive organs.

The function of the posterior crop as an accessory storage organ seems, at first, to be indicated by a comparison of the different stages here considered. Originally a tube of small diameter, it increases in size until it exceeds any of the other organs in capacity. I have never found any precipitate or coagulum in the cavity of this organ, however, so that the mere size of its lumen may not justify one in attributing a storage function to it.

In summarizing it may be said that from the observations upon the four nymphal instars of the cicada it is evident that the digestive organs show an arrangement which is similar in ground plan with that of the adult and also it is similar in many respects with that of other Homoptera which have been described. The complication of digestive organs in the anterior region of the insect is fully established in the earliest nymph and hence is developed at a time previous to that represented in the material here considered. The posterior crop loses its simple tubular character and becomes saccular at some time after the 14 mm. nymph stage.

Since the foregoing part of this paper was written, the paper by Snodgrass dealing with the anatomy of the cicada has come to hand. In this publication the large vesicular organ which

occupies the greater part of the abdominal cavity in the adult insect and which I have called the posterior crop, is considered as a part of the respiratory system. The chief reason for this is the apparent continuity existing between this sac and the first pair of abdominal spiracles. Mr. Snodgrass has been kind enough to demonstrate dissections to me which seem to bear out his contentions. There are, however, some fundamental objections to his position.

In the first place, a "tracheal bladder" or respiratory duct of any kind in an insect should show a lining layer of chitin since the tracheal system of insects arises as an invagination of the primitive ectoderm. In a former paper sections of the "tracheal bladder" of Snodgrass through three different regions were shown and none of these showed any evidence of a chitinous layer. It has been suggested that perhaps this structure attained its respiratory function secondarily and hence might not conform in all structural details to expectations. It is hard to imagine how an entire segment or organ of the digestive tube could undergo such a transformation of function.

In my sections also, I have shown that there are distinct openings at the anterior and posterior ends of this organ, the one at the posterior end leading into a continuation of the digestive tube and that a muscular valve intervened between these two divisions. In view of this evidence it is difficult to conceive of this part of the abdominal contents as having a respiratory function.

It is easy to be deceived as to the continuity of the lumen of the posterior crop with the exterior through these first, abdominal spiracles. In gross dissections there is only the most delicate epithelial membrane limiting this abdominal sac in the region of these spiracles. In my earlier paper a figure was shown covering this point. If a specimen is allowed to become dry, the portion of the wall which is in front of the spiracular opening may easily rupture and then there is an external opening in fact.

Sections show that the spiracle opens into a very small chamber the walls of which break up almost immediately into a number of tracheal tubes which distribute themselves *over the external surface* of the posterior crop. I am therefore, still inclined to question any interpretation which gives this organ a respiratory

function. It certainly becomes modified in later life but it is at all times a part of the digestive system. This condition which is easily observed in sections makes unnecessary the postulation of any secondarily derived function on the part of this organ. The method of gross dissection, then, is inadequate to explain the conditions found.

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EXPLANATION OF PLATES.

INDEX TO FIGURES.

ac—anterior crop.
at—ascending intestine.
dt—descending intestine.
int—internal gland.
kk—coil in descending intestine.
mt—malpighian vessels.
pc—posterior crop.
r—rectum.

PLATE I.

* FIG. 1. Digestive system of cicada nymph of 2 mm. body length, seen from the left side.

FIG. 2. Digestive system of cicada nymph of 14 mm. body length, seen from the left side.
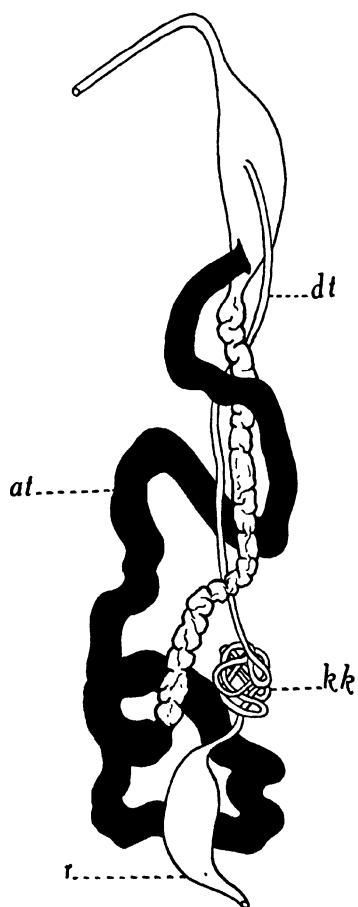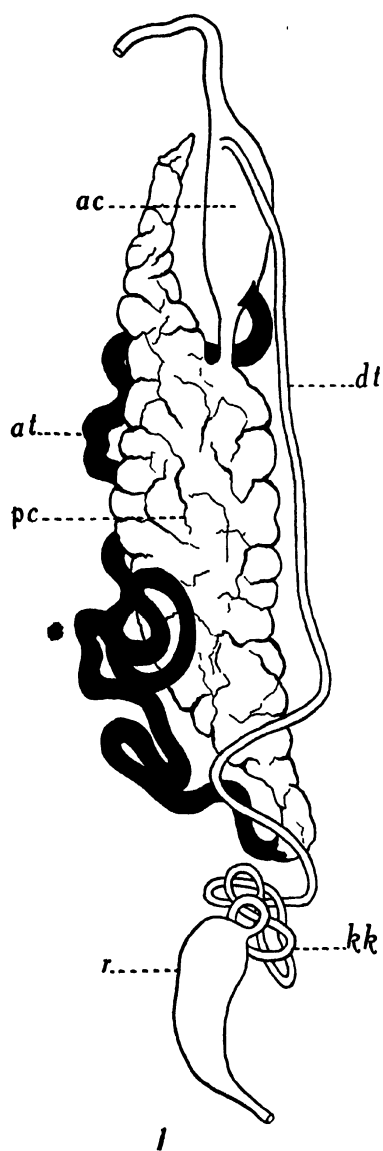
PLATE II.

FIG. 3. Transverse section through the internal gland region of a 2 mm. nymph.

FIG. 4. Transverse section through digestive organs in anterior region of a 7 mm. nymph.

FIG. 5. Transverse section through internal gland region of a 14 mm. nymph

FIG. 6. Transverse section through digestive organs in posterior part of a 7 mm. nymph.
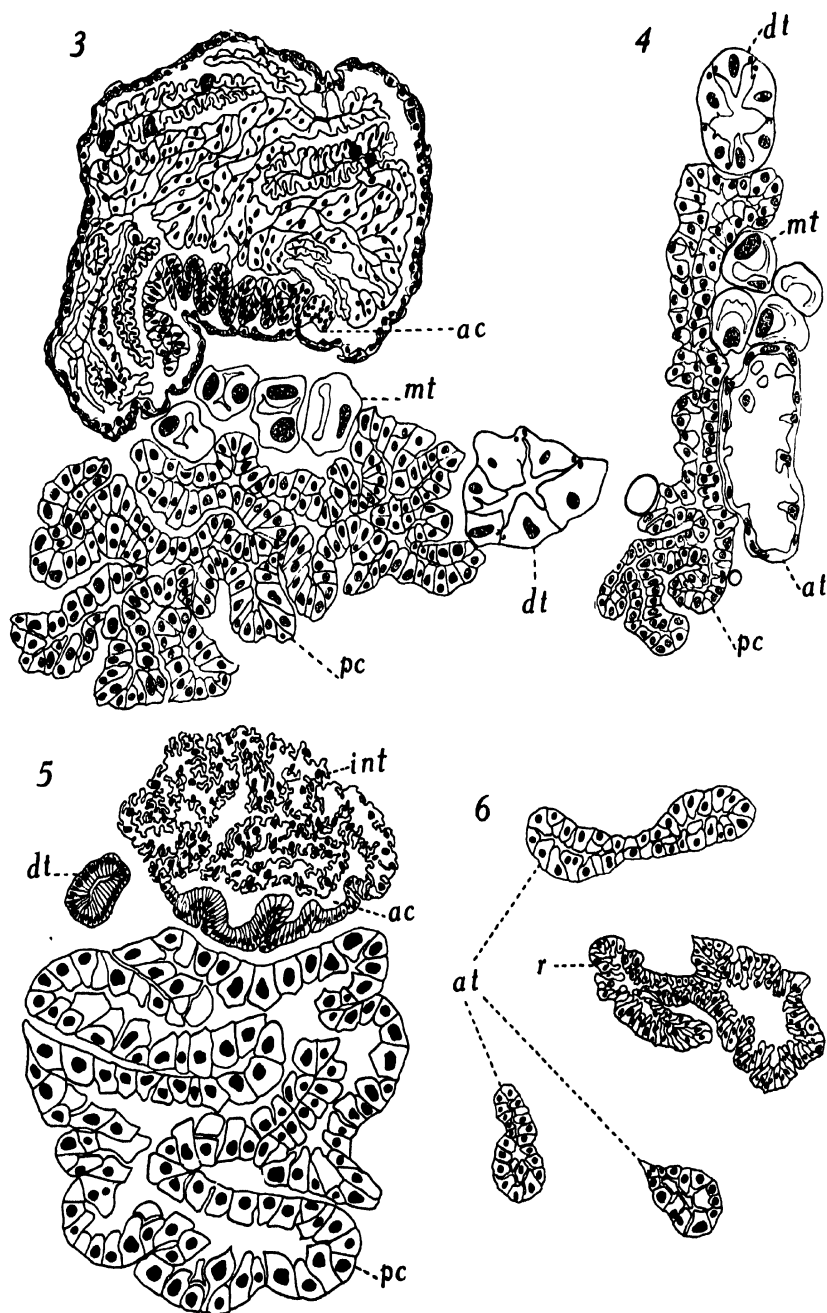
PLATE III.

FIG. 7.   Transverse section through anterior part of digestive organs of a nymph recently hatched.

FIG. 8.   Transverse section through same region but slightly anterior to that represented in Fig. 7.

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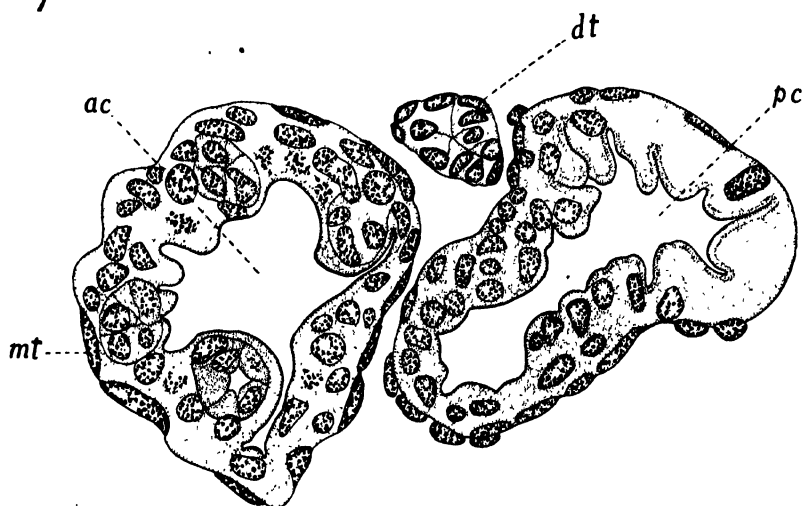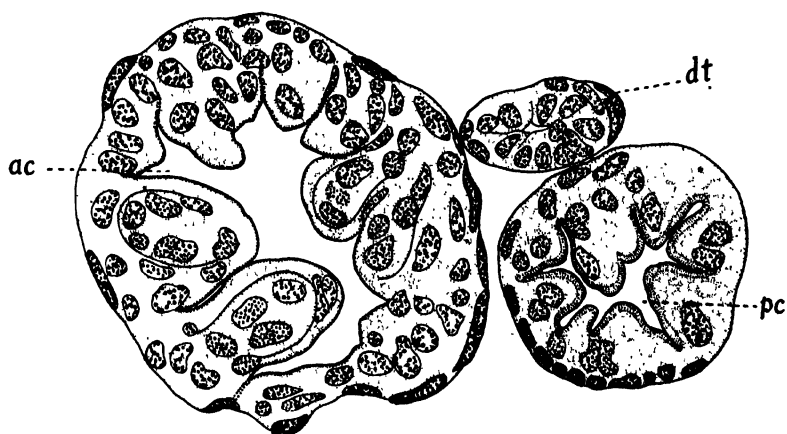
Fig. 9.   Transverse section through digestive organs in mid-body region of a nymph just hatched.

Fig. 10.   Transverse section through posterior part of digestive organs from the same series as Fig. 9.

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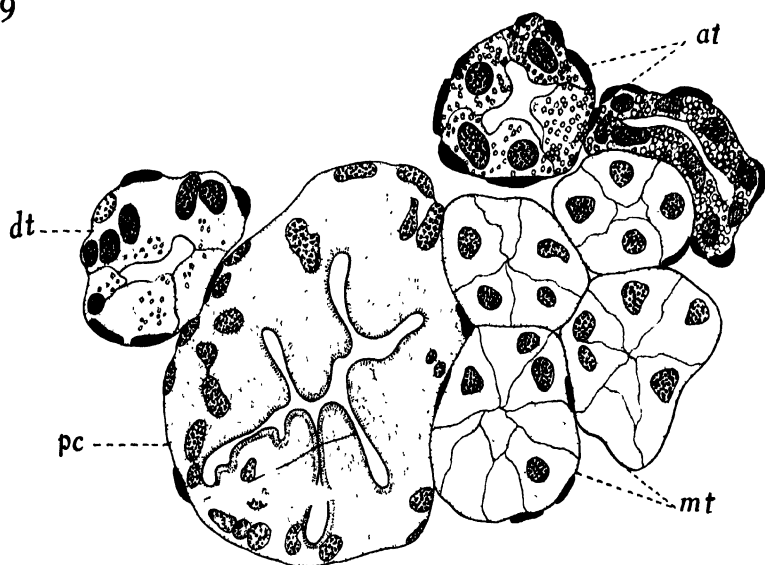
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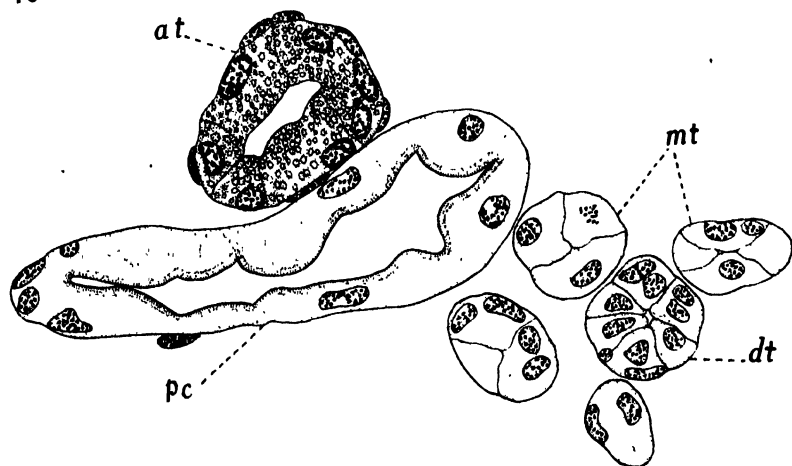
BIOLOGICAL BULLETIN

IODINE AND AMPHIBIAN METAMORPHOSIS.[1]

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Recently the claim has been made that the metamorphosis of urodele larvæ such as axolotl and amblystoma differs from that of anurans in that transformation in the caudate amphibia is independent of iodine and subject only to the influence of the thyroid gland. Experiments are described in this paper which invalidate this conclusion and indeed, render it impossible to understand such a view. The rôle of iodine in metamorphosis is also discussed, and evidence presented showing that the physiologic response (metabolic changes) of mammals to thyroid administration and the metamorphic changes of amphibian larvæ following iodine treatment are probably due to quite different causes and hence not to be compared.

Acknowledgement is due Mr. William Anderson for his labor in iodizing the various compounds employed.

The axolotls came from New Mexico, and were obtained through the courtesy of Mr. J. N. Gladding of Albuquerque.

I. Experiments on Thyroidectomized Axolotls.

The thyroid glands of eight animals were extirpated. One larva was extremely large, measuring over a foot in length. The remaining larvæ averaged seven inches total length. Few technical difficulties are involved in removing the thyroid glands from animals as large as the axolotl. The larvæ were anesthetized in chloretone solutions, placed upon their back under a low-power binocular microscope, and the hyobranchial region strongly illuminated. A median incision was made through the skin and superficial muscles, extending from the posterior edge

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of the hyobranchial region to the symphysis of the mandible. The skin and superficial muscles were pulled apart on either side and pinned down, exposing the geniohyoid and ceratohyoid muscles. The thyroid glands are located in the triangle on each side of the median line, formed by the geniohyoid and ceratohyoid muscles. The large size of the glands and their intimate relation to the blood vessels which traverse the triangle render their location easy. They can be removed from both sides of the animal with but little injury to the blood vessels if first dissected free with a razor-edge needle and gently pulled out with a fine-pointed pair of forceps. Even if the blood-vessels are injured and considerable bleeding occurs, the post-operative effects upon the animal are negligible. It is surprising how much surgical manipulation an axolotl can withstand without showing any post-operative symptoms. Following gland removal the skin and superficial muscles are sutured along the median line. The animals quickly recover from the anesthetic and appear to suffer no ill effects from the operation. Food was witheld for four or five days after thyroidectomy in order to prevent any possibility of tearing the sutures while swallowing.

The thyroidless animals were kept for five months in large concrete tanks through which fresh water ran constantly. The food consisted of worms, insect larvæ, and occasionally, pieces of fresh liver.

Five months following thyroid removal, three animals were injected twice, at five day intervals with eighty milligrams of tyrosine in which two atoms of iodine had been substituted for two hydrogen atoms of the molecule, forming the well-known compound 3–5–diiodotyrosine. The animals metamorphosed within seventeen days following the first injection.

Three control thyroidless axolotls injected with equal quantities of pure tyrosine and 3–5 dibromtyrosine, *i.e.*, tyrosine in which two bromine atoms had been substituted for two hydrogens, failed to transform. Later, one of the controls was injected with a third dose of eighty milligrams of dibromtyrosine but with negative results. Dissection of the metamorphosed iodotyrosine-injected animals showed no trace of thyroid tissue present.

Rogoff and Marine ('17) showed that iodized blood serum accelerates metamorphosis of normal tadpoles, and that the

globulin fraction of the serum contained most of the iodine. It was decided to try injecting iodoserumglobulin into thyroidless axolotls, controlling the experiment by injections of equal amounts of non-iodized globulin. The blood of beef was used.

Three axolotls were each injected twice, at eight-day intervals with 110 milligrams of iodoserumglobulin. Metamorphosis resulted within twenty days following the first injection. One of the axolotls had previously served as a control in the iodotyrosine experiment and had been injected with pure tyrosine. The results were negative and as the larva showed no indications of metamorphosis several weeks later it was utilized in the iodo-serumglobulin experiment. Two control thyroidless animals (previously used as controls in the iodotyrosine work) injected with large amounts of non-iodized serumglobulin failed to transform.

Dissection of the metamorphosed iodoserumglobulin-injected animals showed two of them to have no vestige of thyroid tissue, but the remaining one had a portion of the gland present on the left side, an amount about equal to a third of the entire thyroid. One of the controls was then injected with iodoserumglobulin and metamorphosed twenty-one days after the first injection; dissection of this animal also showed a small nodule of thyroid tissue present. Thus four axolotls were metamorphosed by injections of iodized serumglobulin; two of the animals were completely thyroidectomized, and two only partially. No very marked difference in the time or rate of metamorphosis was noted between the partially and completely thyroidectomized animals. The axolotls possessing remnants of glands were the first to show signs of transformation following injection, but the time difference was not over three days in any case. The fact that both types of animals metamorphosed within a few days of each other may have been due to the large amount of iodoserumglobulin injected. It is probable that partially thyroidectomized animals will be found to respond by metamorphosis to considerably smaller doses than thyroidless animals. Axolotls were not available to test this point.

Considerable difficulty was experienced injecting the globulin intraperitoneally. The solution of the problem while crude was effective. A small incision was made through the ventral body wall sufficiently large to admit the end of a graduated pipette

and the globulin was injected in powdered form. This method because of the ease with which it could be performed was also utilized in the tyrosine experiments. Forced feeding of the animals with the various substances by means of a pipette thrust down the œsophagus into the stomach, might have been as effective as injections, but the animals usually regurgitate some of the material, at any rate this was found to be the case in an earlier experiment where desiccated thyroid tissue was administered by forced feeding.

The pigment pattern characteristic of adult Amblystoma tigrinum is not completely developed in axolotls until several weeks following metamorphosis.

Jensen ('21) metamorphosed axolotls (with intact thyroid apparatus) by injections of iodized casein, iodoserumglobulin, and iodoserumalbumin. He also removed the thyroid glands of axolotls and attempted to metamorphose the larvæ by injections of iodized proteins but his animals died. He concluded that such iodized proteins are highly toxic for thyroidless animals but not for those possessing normal glands. In my own experiments the axolotls withstood the injections of iodized amino-acid and serumglobulin as well as those with pieces of the gland present. There was nothing in the behavior of the animals to indicate that iodized substances are more toxic for thyroidless axolotls than for normal animals. Iodized substances are certainly not more toxic for thyroidless anuran tadpoles than for normal larvæ.

In an earlier experiment than those recorded here three axolotls were thyroidectomized and injected with large doses of iodotyrosine. The animals were kept in ordinary glass aquaria without running water. All of the animals died within ten days following injection. In later experiments the injected axolotls were kept in large concrete tanks, filled to capacity and with fresh water running constantly. None of the animals died. Intraperitoneal injections of large doses of either iodotyrosine or iodoserumglobulin has a marked depressing effect upon thyroidless axolotls; the animals are sluggish, move about very little, refuse food, and show indications of weakness for several days or even a week following injection.[2]

[2] A large axolotl was thyroidectomized and kept for eight months then twice injected at eight day intervals with large amounts of iodized casein. Metamorphosis resulted within twenty-nine days.

The experiment shows beyond doubt that thyroidless urodele larvæ respond to iodized substances in precisely the same manner as thyroidless and pituitaryless anurans. The importance of iodine in the amino-acid and protein molecule in order to render these substances effective in inducing metamorphosis is clearly demonstrated.

II. Experiments on Larval *Spelerpes bislineatus*.

H. H. Wilder years ago ('99) called attention to *Spelerpes* as a favorable object for experimentation. Unlike most urodeles, the larvæ of *Spelerpes* can be obtained at any season of the year and are easily kept under laboratory conditions. The larval life of this form has not been adequately investigated, consequently little is known concerning it. I. W. Wilder ('22) has been engaged for some years in studying the relation of growth to metamorphosis but to date has published only a very brief summary of the results. The data indicate a large range of variation in the size and age of the animals at transformation, with an average larval life of two years.

Eighty animals were used in the experiment, varying in size from 23 mm. to 53 mm. total length. They were separated into four groups of twenty animals each, equal numbers of large and small larvæ being represented. One group of twenty animals was kept in a large aquarium and given plenty of food. The three remaining cultures were subdivided into smaller lots of ten animals each and kept in glass containers in 250 cc. of tap water water through which compressed air bubbled constantly. *Spelerpes* larvæ soon die if kept in small amounts of water unless it is kept cool and well ærated. Twenty individuals were reared in tyrosine solutions representing 120 mg. per 250 cc. of water; another twenty were kept in equivalent solutions of 3-5 dibromtyrosine; twenty more in equal concentrations of 3-5 diiodotyrosine. The animals were fed sparingly during the experiment. Once each week they were removed from the tyrosine solutions and placed in large containers holding 6,000 cc. of water, plentifully supplied with food and allowed to feed for thirty-six hours after which they were returned to the solutions.

The experiment began October 17. Thirteen days from the date the animals were placed in the solutions, two large larvæ of

the iodotyrosine culture showed marked gill and tail-fin reduction. None of the other animals of this or other cultures showed any change. However, by November 5, *i.e.*, twenty days from the first administration of iodotyrosine, all of the larvæ kept in solutions of this substance had metamorphosed. The external gills had disappeared, the gill clefts had closed and the tail fin was completely resorbed. The animals left the water and crawled up the sides of the container.

Examination of the tyrosine and dibromtyrosine cultures showed no indications of metamorphosis. The animals of these cultures were kept in the solutions for a month longer but metamorphosis was not induced. At the close of the experiment exceedingly strong concentrations of tyrosine and dibromtyrosine were employed but with negative results. The larvæ of the normal culture kept in the large aquarium and plentifully supplied with food likewise failed to metamorphose or to show any indications of transformation a month after the animals of the iodotyrosine culture had completed the process.

The results of this experiment are quite clean cut and admit of but one interpretation: It is the iodine within the tyrosine molecule that is responsible for the induced metamorphosis because the tyrosine and dibromtyrosine were ineffective either in weak or strong concentrations when administered over comparatively long periods.

Efforts were made to thyroidectomize the larvæ but without success owing to the extremely small size of the thyroid glands. The thyroid apparatus of *Spelerpes* larvæ of 47 mm. total length does not contain enough of the physiologically active hormone to induce metamorphosis in anuran tadpoles when heteroplastically transplanted. This experiment was attempted by Mr. O. M. Helff of this laboratory but without success.

Judging by the positive results obtained with thyroidless axolotls, it is highly probable that thyroidless *Spelerpes* would react by rapid metamorphosis if injected with, or reared in solutions of iodotyrosine. It is interesting to note that both large and small larvæ metamorphosed within twenty days, though the differences in size were great, varying as they did from 23 mm. to 52 mm. total length. Probably some of the smaller animals were considerably younger than the larger ones, however, it is

impossible to make any definite statements on the point until more is known about the relation of size to age in the larvæ of this urodele.

The fact is well known that the thyroid gland of vertebrates exhibits a remarkable selective action in regard to iodine absorption, taking this element from the blood and synthesizing it into the thyroid hormone by the addition of other substances. In view of this property of thyroid tissue, it is possible, though rather improbable, that in the experiment just cited, the thyroid apparatus of the iodotyrosine-fed animals took up the iodine pouring into the organism and elaborated excessive quantities of the hormone, thus inducing metamorphosis. However, the rapid transformation of both thyroidless frog and salamander larvæ when fed or injected with iodized proteins and amino acids renders such an assumption doubtful.

It is interesting to note that Huxley and Hogben metamorphosed *Salamandra* and *Triton* larvæ by rearing them in dilute solutions of inorganic iodine. But in these experiments the thyroid glands of the animals were intact, hence it is impossible to know whether the action of the iodine was direct or through the mediation of the thyroid.

· III. EXPERIMENTS ON *Amblystoma punctatum*.

It was considered desirable to test the effects of iodo- and bromtyrosine upon the metamorphosis of *Amblystoma* since it was owing to negative results obtained by administration of inorganic iodine to animals of this group that led Uhlenhuth to assert that iodine has no effect upon salamander transformation.

Eighty young larvæ of *Amblystoma punctatum*, averaging 30 mm. total length were divided into four groups of twenty animals each. The larvæ of three groups were isolated in finger bowls containing 50 c.c of water, one animal to each container. The twenty larvæ remaining were reared in large aquaria and fed quantities of tubifex. The animals in the finger bowls received food only at definite intervals and in very small amounts so that they were in a state of semi-starvation during the course of the experiment.

Twenty larvæ received small amounts of diodotyrosine crystals dissolved in the 50 cc. of water in the finger bowls. Twenty

larvæ were fed equal quantities of dibromtyrosine crystals, whereas the remaining twenty animals received no food of any kind and served as controls. Such a culture was considered necessary in order to note any effects starvation might bring about on the progress of metamorphosis since the animals reared on the tyrosine compounds were given very little food.

It should be pointed out here that the animals in the dibromtyrosine solutions received considerably more bromine than the animals of the iodotyrosine cultures received iodine, despite the fact that equal quantities of the two substances were fed to each larva. This is obvious enough since the atomic weight of iodine is 126.92, while that of bromine is but 79.92. Consequently if equal amounts of the two tyrosine compounds were fed or put into solution in given amounts of water as was the case in the present experiment, the number of bromine atoms per milligram of dibromtyrosine would be nearly double the number of iodine atoms per milligram of diiodotyrosine. In all the experiments recorded in this paper this fact has been ignored and the two tyrosine compounds have been administered in equal amounts as though they were chemically equivalent. The experiment began June 22, 1923. On this date none of the small immature larvæ revealed the slightest indication of metamorphosis. June 28 all animals of the iodotyrosine-fed culture showed marked reduction of the gills—three animals had only the stumps remaining. The tail-fin was undergoing reduction. Sand was placed in each finger bowl in order that the animals might crawl out of the water as metamorphosis progressed; this is a necessary precaution, otherwise the animals will drown over night.

Examination of the larvæ reared in the dibromtyrosine solution showed no change; this was also true of the fed and unfed control cultures.

July 4, twelve days from the beginning of the experiment, all of the diiodotyrosine-fed larvæ had completely transformed and left the water. Their gills and tail-fin had disappeared; the branchial clefts had closed and the larvæ had shed their skins but the pigmentation characteristic of the adults of this species had not appeared. Fig. 1, *A*, is a photograph of four iodotyrosine-fed animals. Compare this with Fig. 1, *B*, which
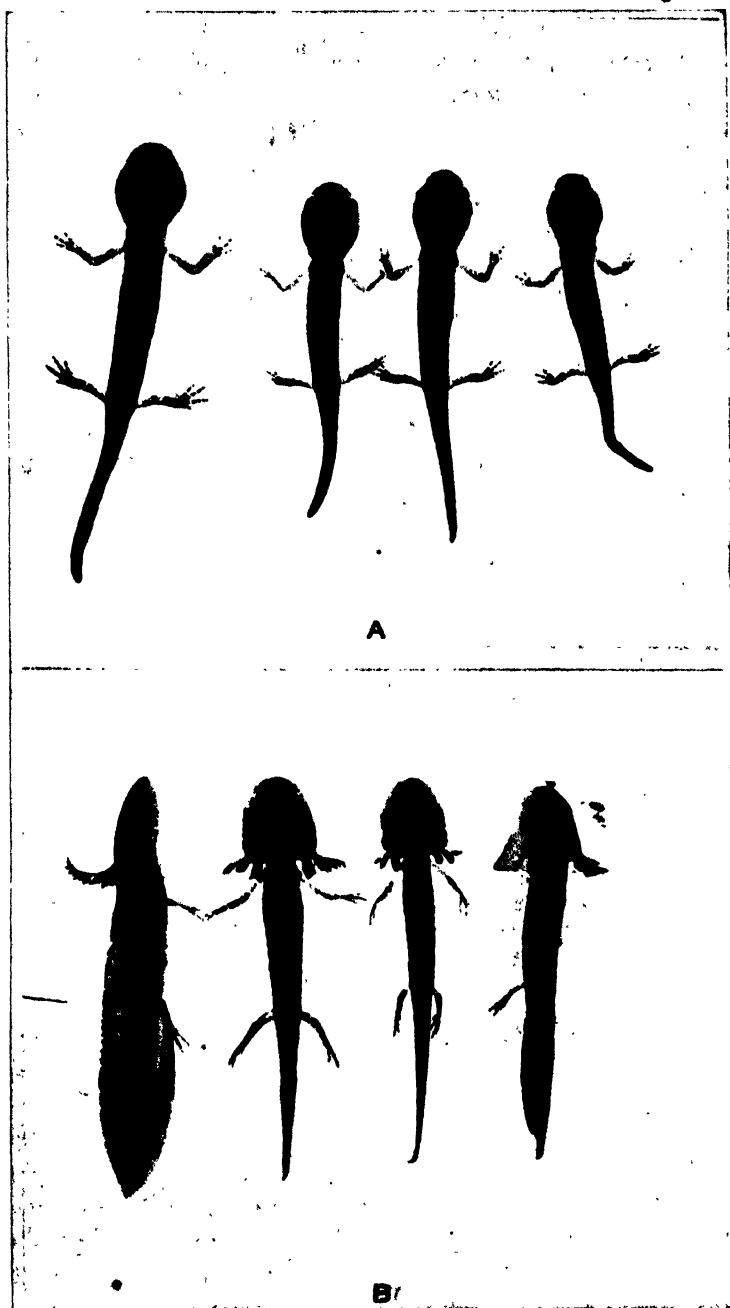
Fig. 1. *A*. Precocious metamorphosis of immature *Amblystoma* larvæ after twelve days immersion in iodotyrosine solution. × 2. *B*. Immature larvæ of *Amblystoma* after twenty-five days immersion in dibromtyrosine solution. × 2.

shows four di-bromtyrosine-fed larvæ. The animals in Fig. 1, *B*, were photographed twenty-five days after the experiment began, hence had been on the dibromtyrosine diet twice as long as the animals of Fig. 1, *A*, had been fed iodotyrosine. The metamorphosed animals were very weak and died within three to five days after metamorphosis. During the twelve days the experiment continued, they were each fed three small worms, the animals of the other cultures (except one) received the same.

The dibromtyrosine culture was continued twenty-six days after transformation of the iodotyrosine culture had occurred, *i.e.*, thirty-eight days from the date of first feeding. At the end of this time eleven animals metamorphosed, the remaining larvæ did not transform. The culture was abandoned July 31. The fed and unfed control groups were also given up at this time.

It is an interesting fact that none of the control animals of either starved or fed cultures metamorphosed during the thirty-eight days of the experiment, whereas eleven larvæ of the dibromtyrosine culture did transform. The experiment indicates that unfed salamander larvæ can be forced to metamorphose if reared in very strong solutions of dibromtyrosine over long periods. The bromine in the tyrosine molecule is thus seen to be not entirely inert as regards metamorphosis though it cannot be compared with iodine. For instance, diiodotyrosine solutions of about one-half the concentration of the dibromtyrosine, metamorphosed salamander larvæ of equal size and developmental stage in a period ranging from seven to twelve days, whereas the much stronger bromtyrosine solutions caused about two-thirds of the larvæ of the culture to transform between the thirtieth and thirty-ninth day.

In an earlier paper ('22) the writer called attention to the fact that the hind legs of thyroidless and pituitaryless tadpoles reared in strong dibromtyrosine solutions grow larger than the limbs of like animals on an algæ diet but that such animals do not metamorphose. Here again the evidence indicates that the bromine ion is not entirely passive since it does stimulate to a slight degree the growth of the tadpole's hind limbs. But the degree of activity of the bromine in the tyrosine molecule is not comparable to that of iodine. The following experiment clearly shows the great difference in activity between the two substances. Three sets of
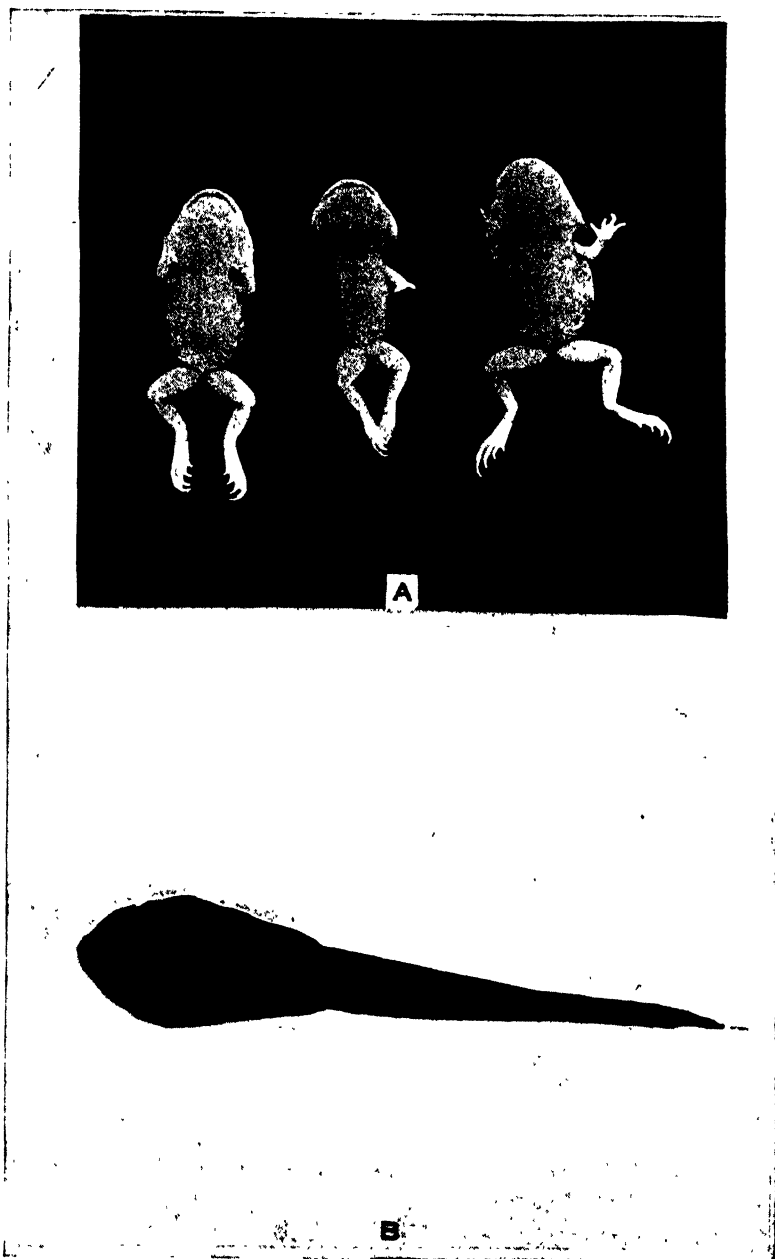
Fig. 2. A. Immature *Rana clamitans* larva metamorphosed by immersion in iodotyrosine solution for fifteen days. B. *Rana clamitans* tadpole reared for twenty days in dibromtyrosine solution.

thyroidless and pituitaryless *R. sylvatica* larvæ were used. One set of animals was reared in tyrosine solution, another in iodotyrosine, a third set in dibromtyrosine of twice the strength of the iodotyrosine solution. No food of any kind was given. The iodotyrosine cluture metamorphosed within fifteen days, the animals of tyrosine and dibromtyrosine culture showed no indications of metamorphosis after forty-three days. However, the larvæ reared in the dibromtyrosine solutions had well developed hind legs averaging 7 mm. total length as compared with an average of 3.5 for the animals of the tyrosine culture. Since no food of any kind was given the increase in limb growth was probably due to a slight influence exerted by the bromine ion.

The result of this experiment upon *Amblystoma punctatum* larvæ is identical with that obtained with *Spelerpes*, and shows clearly that it is the iodine in the tyrosine molecule that is responsible for the precocious metamorphosis. The rapidity with which the metamorphic response to iodotyrosine is evoked in salamander larvæ, would seem to indicate that the action of the iodized amino-acid is directly upon the cells and tissues of the organism and not necessarily through the intermediation of the thyroid gland. It will be recalled, that young thyroidless axolotls (*Amblystoma tigrinum*) transform following injections of iodotyrosine and it is probable that thyroidless *Amblystoma punctatum* would react likewise. However, no experiments were made to test this point.

The writer now has under way a series of experiments upon the metamorphosis of thyroidectomized salamander and anuran larvæ in which various iodized proteins and amino acids are fed and injected. The results will be communicated later.

IV. Experiments on *Rana clamitans* Tadpoles.

The larval life of the green frog, *Rana clamitans*, extends over a year (370–400 days according to Wright, '14) and in some individuals is prolonged two years before metamorphosis occurs. This form offers exceptional opportunities for experimentation because of the long duration of larval existence, and is especially valuable for use in investigations on metamorphosis where the feeding method is employed. Tadpoles of all sizes are obtainable at any season of the year and when brought to the laboratory

readily adapt themselves to the changed environment. Animals captured in the autumn generally pass the winter and spring as tadpoles.

September 13, 1922, a large number of larvæ were collected and eighty tadpoles of approximately equal size and developmental stage were selected and divided into four groups of twenty individuals each. One group was fed tyrosine, another ordinary tadpole food such as spirogyra and insect larvæ, the third lot received dibromtyrosine and the fourth group diiodotyrosine. A fifth culture of animals, forty in number, of varying size and developmental stage was kept in large glass aquaria and fed quantities of algæ. Each culture except the fifth was further subdivided into lots of five tadpoles each, and placed in 250 cc. of tap water containing 120 mg. of the tyrosine compounds. Each evening the animals were transferred to large jars containing 10,000 cc. of tap water and plentifully supplied with algæ. This procedure was considered necessary in order to rule out the starvation factor since tadpoles can not exist indefinitely on tyrosine alone.

The animals obtained the tyrosine compounds by two methods: through the alimentary tract, and by absorption through the skin. Most of the tyrosine goes into solution after a short time, hence the larvæ probably obtain most of this substance through the skin. This is certainly the manner in which *Spelerpes* larvæ obtain the tyrosine and its iodized and brominated compounds.

Table I. gives the average measurements of the animals at the beginning and end of the experiment. The figures are averages based upon measurements of fifteen animals of each culture.

TABLE I.

	Algæ-Fed.		Tyrosine.		Dibromtyrosine.		Diiodotyrosine.	
	Total Length, mm.	Hind Legs, mm.	Total Length, mm.	Hind Legs, mm.	Total Lengtn, mm.	Hind Legs, mm.	Total Length, mm.	Hind Legs, mm.
Sept. 13.....	51.2	3.9	52	3.5	52.2	3.8	51.5	3.5
Oct. 8.......	52.2	3.9	52.5	3.8	52.5	3.9	Advanced stages of metamorphosis	

Thirteen days from the beginning of the experiment a marked

difference was observed between the iodotyrosine-fed animals and those of the other cultures. The tyrosine and dibromtyrosine-fed tadpoles showed no changes either in regard to growth or metamorphosis from the algæ-fed controls, whereas the iodotyrosine-fed animals appeared thin and emaciated; the hind legs had increased in length also the skin of the pectoral region where later the fore-legs appear, was undergoing autolysis.

October 3, four of the iodotyrosine-fed tadpoles had fore legs and frog mouths. The hind legs of all of the animals had markedly increased in length, and the tail was undergoing resorption. In several individuals large patches of skin over the region of the fore-limbs was totally destroyed by autolysis. The animals of the remaining cultures showed no change.

October 6, the animals of the iodotyrosine culture were all in advanced stages of metamorphosis and many were dying; only one animal remained alive on October 8. Table I. gives the measurements of the tadpoles of the various cultures at the close of the diiodotyrosine experiment, twenty-three days after the date of first feeding.

The remaining cultures were continued until October 30. The tyrosine and dibromtyrosine-fed tadpoles were then taken off the tyrosine diet and fed algæ. The larvæ were measured November 13 two months after the beginning of the experiment but no indications of metamorphic change was observed. It is quite evident that insofar as the metamorphosis of the green frog is concerned, tyrosine and dibromtyrosine are ineffective, even when administered in large quantities. These results are in agreement with those obtained in similar experiments on thyroidless axolotls and thyroidless and pituitaryless anuran tadpoles (Swingle, '22).

<center>DISCUSSION.</center>

The experiments described in this paper and elsewhere on the administration of iodine, iodized amino-acids and proteins to thyroidless and pituitaryless anuran larvæ and to thyroidless axolotls demonstrate that other forms of iodine than that peculiar to the thyroid hormone (thyroid iodine) possess the power of inducing amphibian transformation. This property of iodine is apparently unique, since so far as known at present it is not shared by other substances, and is inherent in the iodine atom when

organically combined in a certain way. The type of combination is not necessarily that characteristic of thyroid iodine because a large number of iodine compounds have the power to bring on metamorphosis in thyroidless amphibians—even elemental iodine itself. That elemental iodine is utilized within the organism as iodine per se for any purpose seems improbable because if one compares the effect of administering inorganic iodine and various organic preparations to thyroidless tadpoles, it becomes evident that the physiologic activity and metamorphosis-inducing properties of the organic preparations are superior to elemental iodine. Furthemore, the accelerating effect upon metamorphosis of the organic iodine compounds is less than that of the thyroid extract itself. However, the important thing is not the speed with which iodine compounds induce metamorphosis in comparison with the thyroid hormone itself, but the fact that iodine other than thyroid iodine induces the metamorphosis of thyroidless animals. The crux of the problem in regard to amphibian metamorphosis, is to find out just what it is in tyrosine, serumalbumen, serumglobulin, casein, tyramine and probably a host of other amino acids and proteins which when iodine is added increases so remarkably the metamorphosis-inducing powers of this element. Why, for example, does the linking of iodine to the third and fifth carbon atoms of the benzene ring in the tyrosine molecule transform this inert (so far as metamorphosis is concerned) amino acid into a highly active agent. What is it in casein, albumen, globulin or tyrosine that raises so greatly the reactive powers of iodine?

There can be little doubt that elemental iodine when it induces the transformation of thyroidless and pituitaryless tadpoles combines either with the proteins of the algæ fed along with it, or within the body of the tadpole after absorption through the skin or alimentary tract, but it doesn't combine with anything produced by thyroid tissue because there was none present nor .had there ever been any present in the case of the thyroidless ,anurans.

Hirschler ('22) precociously metamorphosed tadpoles by inserting small pieces of elemental iodine into the body of the larvæ. However, since the animals possessed intact thyroid glands their activity can not be ruled out in this experiment,

because the metamorphosis may have been due to increased activity of the thyroid due to increased iodine supply. On the other hand the absorbed iodine may have combined with other than thyroid proteins thus inducing metamorphosis. Either or both of these possibilities may have been realized in Hirschler's experiment, consequently work involving iodine administration should be performed only on thyroidectomized larvæ since only this type of experiment will shed any light upon the rôle of iodine in amphibian metamorphosis. This is especially true of neotenous forms like axolotl; no work on the rôle of iodine in the metamorphosis of this form should be regarded as conclusive unless performed upon animals from which all trace of thryoid tissue has been removed.

Thyroid conditions in axolotl are very peculiar (Swingle, '22) and vitiate the results of experiments done on animals with intact glands. The New Mexican strain of axolotl has perfectly developed thyroids, the vesicles filled to capacity with the physiologically active hormone yet the secretion is apparently unable to escape into the blood stream in sufficient quantities to transform the animal. This is demonstrated by heteroplastic thyroid transplants. An axolotl thyroid is sufficient to metamorphose thirteen normal, thyroidless and pituitaryless anuran tadpoles when grafted, but left intact within the axolot's body is incapable of initiating metamorphosis. [1] It is obvious that in this form we are dealing with a thyroid mechanism which selects, stores and transforms the iodine of the animal's food and water into the thyroid hormone but fails to release the elaborated product. Consequently, if axolotls are fed elemental iodine they do not metamorphose, and for exactly the same reason, they fail to transform under the ordinary dietary regime—the iodine is picked up by the gland and synthesized but not released. It is probable that feeding organic iodine preparations would give similar results. On the other hand it is erroneous to conclude from such an experiment that iodine has no ·influence on axolotl metamorphosis. Our experiments on both thyroidectomized. and partially thyroidectomized axolotls show quite clearly that iodized amino acids and proteins promptly metamorphose these

[1] This experiment was performed by Mr. Karl Mason, of this laboratory, and reported at the Boston meeting of the American Society of Zoölogists, 1922.

animals, and that the amino-acid and protein without the iodine in the molecule are inert.

Hirschler ('22) metamorphosed axolotls (the European strain which rarely spontaneously transforms) by implanting iodoform paste within the body cavity. However, in this experiment the glands were intact so it may be that the iodoform merely served to stimulate the thyroid mechanism to release its stored hormone thus inducing metamorphosis. It will be recalled that Kaufman ('18) metamorphosed axolotl by injections of salicylic acid. So far as is known salicylic acid has no influence on metamorphosis, the effect of injecting the substance into axolotl was to stimulate the secretory activity of the thyroid apparatus in some way. Anyone not familiar with thyroid conditions in axolotl might conclude that salicylic acid per se was the metamorphosis-inducing agent, whereas probably nothing could be farther from the real facts of the case.

The New Mexican strain of axolotl if removed from its native habitat to New Haven soon undergoes spontaneous metamorphosis. Why? Certainly not because the railroad journey exerts any mysterious metamorphosis-inducing power, but probably because the changed food, water, the jolting and confinement incident to the trip acted as a stimulating agent thus releasing the thyroid hormone from the gland vesicles thereby causing transformation.

Where the thyroid apparatus is left intact the fate of the substances fed or injected into an amphibian larva is problematical. If an effect upon metamorphosis is produced it is impossible to determine whether the effect is due directly to the substance itself, or indirectly through intermediation of the thyroid unless the work is checked by repeating it upon thyroidless forms. An excellent illustration of this statement has been furnished by Allen ('20). Allen observed that transplantation of the anterior lobe of the pituitary into hypophysectomized tadpoles (but possessing a rudimentary and functionless thyroid apparatus) metamorphosed the animals, whereas transplantation of the pituitary gland into thyroidectomized larvæ had no such effect. The pituitary secretion had no direct influence upon metamorphosis but acted indirectly by stimulating the rudimentary thyroid into functional activity. Any one working with

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normal tadpoles with intact thyroids uncontrolled by thyroidless animals might well have concluded from the results of such an experiment that pituitary tissue exerts a direct stimulus to metamorphosis and is thus equivalent to the thyroid.

Recently ('22) Romeis claims to have isolated an absolutely iodine-free substance from the thyroid gland which when administered to frog tadpoles exhibits all of the physiologic effects of thyroid gland tissue. The writer is skeptical of the validity of this claim and for several reasons: (1) All active substances so far isolated from the thyroid contain large amounts of iodine. Thyroxin the active principle contains sixty-five per cent. of iodine as an integral part of the molecule; and it is known that thyreoglobulin containing no iodine is physiologically inert; (2) Experiments on tadpoles have shown (Rogoff, '18-19) that blood coming from hyperplastic thyroid glands with extremely low iodine content fails to induce tadpole metamorphosis; (3) To date the only substances known to metamorphose thyroidless tadpoles are thyroid or iodine in some form—presumably in the last analysis organically combined. While writing this paper the writer came across a second communication from Romeis which practically amounts to a retraction of his earlier claims. He found that iodothyrine and iodothyroglobulin will induce the metamorphosis of tadpoles in dilutions of one in a million; thyroxin produced the same effects on growth and metamorphosis in dilutions of one in ten million consequently says Romeis: These figures suggest that effects (on tadpoles) from so-called iodine-free materials from the thyroid may have been contaminated with minute amounts of thyroxin.

There can be little doubt that this is the real explanation of the results obtained by this investigator with so-called iodine-free substances from the thyroid.

There seems to exist a fundamental difference between mammals and amphibians in regard to their physiologic response to thyroid and iodine administration. This difference is not sufficiently understood by those who attempt to compare these two verebrate groups. The following experiment of Kendall ('19) is an excellent illustration of the point: He found that injections of pure thyroxin into mammals is followed by a very definite and marked physiologic response. But when the hydrogen of the

imino group in the thyroxin is replaced with acetyl, the substance
loses its physiologic activity and there follows no demonstrable
effect upon the metabolic rate.   This emphasized the importance
of the imino group in thyroxin (in so far as the metabolic effect
upon mammals is concerned) and minimizes the importance of
the iodine in the molecule.   Bearing in mind the effect of iodine
administration upon tadpole metamorphosis, Kendall was led
to try the acetyl derivative of thyroxin on tadpoles, for if meta-
morphosis depends only upon the increase in the basal metabolic
rate of the larvæ then thyroxin should increase the rate of meta-
morphosis but the acetyl derivative involving the imino group
should not.   If, however, iodine alone is concerned in accelerating
metamorphosis, then both thyroxin and the derivative should
affect the transformation.   Kendall found that both thyroxin
and the acetyl derivative would induce a rapid metamorphosis
of the bull frog tadpole.   Kendall's conclusion was that thyroxin
appears to have two separate and distinct functions: the effect
upon the metabolic rate which is brought about by the CO-NH
groups within the molecule; and the physiological changes in-
volved in the metamorphosis of the tadpole due to the iodine
contained in the molecule.   Our own experiments have demon-
strated that this action of iodine is not specific to thyroxin, but
can be obtained in thyroidless amphibians (though the effects
are not so rapid, and the amounts administered must be larger)
by a large number of other iodine compounds and by adminis-
tration of elemental iodine itself.

Kendall's experiment sheds considerable light on the reason
for the conflicting results obtained by investigators working with
iodine, iodized proteins and amino acids on mammals, and the
students of amphibian metamorphosis.   In mammals the criterion
employed for testing the phsiologic action of iodine and thyroid
upon the organism is the effect upon metabolism as indicated by
changes in the nitrogen excretion, CO_2 elimination and oxygen
consumption; in amphibians the criterion has been the rate and
degree of the degenerative and regenerative processes incident
to metamorphosis.   However, it is becoming clear that the two
types of physiologic response are not in the same class and hence
not to be compared because they owe their origin to different
causes.   The unique effects of thyroid or thyroxin upon the meta-

bolic rate is due to the specific chemical structure of the thyroxin molecule particularly the CO–NH group, whereas metamorphic response of amphibians is dependent upon a peculiar property of iodine in certain types of combination, although not necessarily that characteristic of thyroid iodine, *e.g.*, iodized amino acids and proteins. Further evidence of the difference as to cause between metabolic changes in mammals and amphibian metamorphosis is furnished by the acetonitrile test where iodized substances shown to be specific in accelerating metamorphosis completely fail to simulate the thyroid function in protecting mice against the lethal effects of actonitrile.

Hunt and Seidell ('09) made the interesting observation that feeding thyroid tissue to white mice greatly increases the resistance of these animals to lethal doses of acetonitrile, and that the efficiency of the gland seems dependent upon its iodine content. They concluded that the increased resistance of the mice to the poison was due to the changed metabolism of the animals following thyroid feeding, the metabolic change preventing the acetonitrile from breaking down into its posionous product hydrocyanic acid. This assumption was based upon the fact that thyroid feeding does not raise the resistance of mice to lethal doses of hydrocyanic acid itself.

Koch ('13) and Miura ('22) found that iodized amino acids such as diiodotyrosine iodotryptophan and tetra-iodohistidine when administered to mice fails to increase their resistance to acetonitrile.

Strouse and Voegtlin ('09–'10) failed to observe any thyroid-like effect on the nitrogen metabolism or on the blood pressure of normal dogs, nor was there any favorable effect on the condition of myxedematous and cretinous mammals following administration of iodized amino acid. Other investigators have tried in vain to obtain thyroid effects on mammals (metabolic changes) by the use of various iodized substances; tri-iodo-imidazol and, iodophenylalanie have also proven ineffective.

If, however, we bear in mind the results of Kendall's experiment, it becomes clear why iodized amino acids, *e.g.*, iodotyrosine, give negative results when administered to myxydematous and cretinous mammals, and positive results when fed to thyroidless amphibian larvæ. In the latter group the metamorphic response

is due to the iodine in the molecule, in the former group the meta-bolic response depends upon something else, *i.e.*, the CO–NH group within the thyroxin molecule. This brings us to the consid-eration of another point, *i.e.*, the possibility of substituting other halogens for iodine and obtaining the same effects upon meta-morphosis.

Kendall ('18) as a result of his investigations of the unique effects of thyroxin upon the metabolic rate of mammals, was led to conclude that insofar as the physiologic effect upon mammals is concerned, possibly other halogens could be substituted for the iodine of the thyroxin molecule without greatly changing the physiological properties of the thyroxin. His conclusion follows: "In regard to the relation of iodine to the activity of thyroxin, the presence of iodine in the compound must exert some influence, and it seems not improbable that the presence of iodine renders the active groups more reactive. In the absence of iodine it would take a greater working pressure to bring about its reaction. The substitution of iodine by hydrogen or chlorine or bromine would undoubtedly be followed by an alteration in the degree of reactivity of the substance but its gross chemical nature and properites would not be altered thereby."

Whether or not other halogens can or cannot be substituted for the iodine of the thyroxin molecule and this substance still retain its physiological activity in mammals is an open question, at any rate there are no experimental data tending to answer the ques-tion one way or the other except possibly the work of Ostwald and von Cyon who observed that thyreoglobulin containing no iodine was physiologically inert, whereas this substance gives all the physiological effects of thyroid tissue when iodine is present. However, this may be, it is clear that insofar as amphibian meta-morphosis is concerned other halogens such as bromine can not be substituted for iodine. The experiments upon thyroidless axolotls and anuran larvæ where 3-5 dibromtyrosine was em-ployed, demonstrates the futility of endeavoring to substitute bromine for iodine with hope of affecting metamorphosis. It is merely quibbling to say that bromine would probably be just as effective as iodine providing the organism possessed a mechanism for utilizing this halogen in the way the thyroid utilizes iodine to elaborate its hormone, because in our experiments the animals

fed iodotyrosine and iodoserumglobulin had no vestige of thyroid tissue present, consequently had no mechanism for iodine utilization.

No one has ever shown that in the absence of thyroid glands, other tissues of the organisms have the power of functioning vicariously for the thyroid, and synthesizing its active hormone. If this possibility were true then why is it that mammals with atrophied or degenerate thyroids are quite unable to utilize iodine or iodized proteins and amino-acids. If other tissues of vertebrates besides the thyroid glands possess the power to manufacture the thyroid hormone it is strange that this power should be present in amphibia yet lacking in mammals. The truth of the matter is that amphibian metamorphosis depends upon a peculiar property inherent in the iodine atom when combined in certain ways.

Some investigators have claimed that the pituitary can function vicariously for the thyroid when the latter is absent, hence it might be said that in thyroidless amphibians the pituitary gland may synthesize the iodine into the chemical complex characteristic of the thyroid hormone. Aside from the total lack of evidence that the pituitary can function vicariously for the thyroid in thyroidless forms, the experiments of Allen ('19) are of interest in this connection. Allen extirpated both the thyroid and the pituitary gland of frog embryos and later fed the tadpoles with starch iodide. The animals underwent a precocious and nearly complete metamorphosis before death ensued, clearly demonstrating that iodine is as effective in inducing transformation in tadpoles lacking both thyroid and pituitary as in larvæ with only the thyroid missing.

SUMMARY OF CONCLUSIONS

1. Thyroidectomized, partially thyroidectomized, and normal axolotls are readily metamorphosed by intraperitoneal injections of iodotyrosine and iodoserumglobulin and iodocasein.

2. Thyroidectomized, partially thyroidectomized and normal axolotls do not metamorphose when injected with large quantities of pure tyrosine, 3-5 dibromtyrosine (two atoms of bromine in the molecule) and non-iodized serumglobulin.

3. Larval *Spelerpes bilineatus* readily metamorphoses in strong solutions of 3-5 diiodotyrosine, but do not transform in equivalent concentrations of tyrosine and 3-5 dibromtyrosine even when kept in such solutions over comparatively long periods.

4. *Rana clamitans* tadpoles with from six to eight months of larval life remaining (*i.e.*, passing the winter as tadpoles) were metamorphosed within twenty days by rearing the animals in strong concentrations of 3-5 diiodotyrosine and feeding them with this substance. Control larvæ of similar age and developmental stage reared in equivalent solutions of 3-5 dibromtyrosine and fed quantities of the compound failed to transform.

5. The experiments are clean cut and admit of but one interpretation; it is the iodine in the amino acid and protein molecule that is responsible for amphibian metamorphosis. Why the linking of iodine to the third and fifth carbon atoms of the benzene ring of the tyrosine molecule should so greatly increase the reactive powers of the iodine is unknown.

6. Iodine other than thyroid iodine is effective in inducing the metamorphosis of thyroidless urodele and anuran larvæ. There is no evidence that any other tissue of the vertebrate organism has the power to function vicariously for the thyroid in the latter's absence.

7. Bromine has little or no influence upon amphibian metamorphosis and can not be substituted for iodine. The substitution of two bromine atoms for two hydrogen atoms (the third and fifth) of the tyrosine molecule fails to change this substance into a metamorphosis-inducing agent.

8. The physiologic responses (metabolic changes) of mammals to thyroid administration are due to the CO–NH group within the thyroxin molecule; amphibian metamorphosis to the iodine in the molecule. Consequently thyroidless animals of these two vertebrate groups are hardly comparable in regard to their response to iodine. Only thyroid iodine containing the CO–NH group is effective in thyroidless mammals whereas iodine other than thyroid iodine will metamorphose thyroidless amphibian larvæ.

9. Data are presented showing why experiments on amphibian larvæ devised to test out the effect upon metamorphosis by feeding or injecting various substances are unsound unless per-

formed upon thyroidless animals or at any rate controlled by identical experiments upon thyroidectomized individuals.

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SOME NOTES ON THE FERTILIZATION REACTION IN ECHINODERM EGGS.

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The following observations make no claim to novelty but are perhaps worthy of record as additional evidence on certain matters. They are concerned solely with the fertilization reaction, that is to say, with the activation of the egg, and not with cleavage. Activation and cleavage must, I think, be regarded as distinct processes.

The observations were made in May, 1922, at the Hopkins Marine Station, Pacific Grove, California. I am indebted to the director, Dr. W. K. Fisher, for the privilege of working at the station and for his unfailing promptness in supplying me with everything necessary for my work.

1. Materials and Methods.—The eggs used were those of the Pacific coast sea-urchins, *Strongylocentrotus franciscanus* and *purpuratus*, and of the starfish, *Patiria miniata*. The latter is the *Asterina* of Loeb. The eggs of the urchins were obtained by removing the oral portion of the test, leaving the ovaries in the aboral portion. All-other viscera and tissues were then removed from the latter and it was rinsed several times with sea-water. Upon standing for a short time, those portions of the ovaries which are ripe break down, releasing the eggs, which may then be removed with a pipette. The urchins were evidently past their prime at the time during which I worked with them as only small portions of the ovaries contained mature eggs. These seemed, however, to be entirely normal in most cases, and gave a high percentage of fertilization membranes and cleavage.

The eggs of *Patiria* employed were normally shed eggs. It was found that when these starfish are spread out on a table, a considerable number of them (presumably those that happen to be ripe) will begin to shed eggs and sperm and continue this for three or four hours. It may be noted in passing that the normally shed eggs of *Patiria* are fully mature, are immediately

fertilizable as they exude from the genital pores, and yield 100 per cent. fertilization membranes, cleavage, and larvæ. This agrees with observations on other starfish. As is well known, and this is also the case in *Patiria*, starfish eggs obtained by shaking or mincing the ovaries are not mature, but must stand in sea-water for some time before they attain a fertilizable condition.

In the following account a number of statements are made concerning viscosity differences. These have been determined in the following manner. The eggs are placed on a slide in a drop of sea-water without cover. Low power was used (Leitz objective 2, ocular 3). Any egg to be investigated was rapidly pushed with a needle to the periphery of the drop and then into a small evagination of the periphery where it is held by surface tension. It was then punctured or cut with a needle and the rate and readiness with which the egg cytoplasm flows out under the pressure of surface tension as well as the duration of retention of cuts and gashes furnish a relative measure of the viscosity of the cytoplasm. The needle used was a fine steel needle thrust into a wooden handle and operated by hand. The method is somewhat crude but has the advantages that it is rapid and almost entirely objective.

2. Viscosity of the Unfertilized Mature Egg.—The unfertilized egg in all three species consists of a slightly viscous cytoplasm inclosed in a definite membrane of considerably greater consistency than the cytoplasm. This membrane, which may be designated the vitelline membrane (it has also been named plasma membrane and egg membrane) is probably a colloidal gel. It is more delicate in the sea-urchin than in the starfish egg.

These facts have been determined as follows. When the sea-urchin egg (either species) is held by surface tension and punctured with a needle, the cytoplasm rushes out with almost explosive force and the whole egg very rapidly disintegrates. In this disintegration the vitelline membrane is also involved so that I at first thought such a membrane was absent. However, if the eggs are rapidly pushed back into the drop when they are partially disintegrated, egg fragments of various sizes are obtained. On the surface of such fragments wrinkles are always observable. It is evident that such wrinkles must be located in a surface membrane of greater consistency than the cytoplasm,

in fact, of solid consistency, as fluids do not exhibit permanent wrinkles. These wrinkles on egg fragments are illustrated in Figs. 10 and 13. In only one case did the entire membrane persist after disintegration of the cytoplasm; this case is illustrated in Fig. 16. The membrane is collapsed and wrinkled.

In the *Patiria* egg, when punctured as just described, the cytoplasm flows out invariably leaving the membrane behind. This is illustrated in Figs. 19 to 25. From the fact that the membrane always persists in the starfish egg after disruption of the cytoplasm and rarely so persists in the urchin egg, I have drawn the conclusion that the vitelline membrane of the former egg is firmer, tougher, and probably thicker than in the latter egg. This difference is further evidenced by the fact that the empty membrane in *Patiria* retains its former shape better than in the urchins as may be seen by comparing Figs. 16 and 19.

The consistency of unfertilized eggs has been previously described by a number of investigators. The first detailed description of the viscosity conditions in unfertilized eggs seems to have been that of Herbst ('93). Herbst noted that if pressure is applied to unfertilized sea-urchin eggs, the contents flow out and a fine membrane is sometimes left behind to which bits of protoplasm still cling. He therefore concluded that the surface layer of the egg possesses a greater consistency than the remainder of the egg but is not definitely separated from the latter. Lillie ('06) notes that the *Chætopterus* egg is semifluid and surrounded by a delicate membrane. Heilbrunn ('15) states that the cytoplasm of the unfertilized *Arbacia* egg is "typically fluid" and inclosed in a membrane described as being "a protein gel" and possessing "a certain degree of rigidity." Chambers ('17a) finds that the protoplasm of the unfertilized eggs of *Arbacia*, *Asterias*, *Echinarachnius*, *Cerebratulus*, and *Fucus* is a "hyaline fluid" of "very slight consistency" while the surface layer is "very dense in consistency as compared with the cell interior into which it merges insensibly." Chambers has also noted the greater delicacy of the membrane of the urchin egg than of the starfish egg ('21b). Heilbrunn ('20b) describes the *Cumingia* egg as "a mass of fluid protoplasm surrounded by a rigid membrane." Seifriz ('18, '20) finds that ripe *Fucus* eggs are decidedly viscous with a wall consisting of a very rigid hyaline gel; he also describes

the cytoplasm of *Tripneustes* and *Echinarachnius* eggs as of about the consistency of glycerine. The cytoplasm of the eggs which I have investigated seems to me to correspond to 4 or 5 in Seifriz's scale ('20, p. 364). The existence of a membrane around the unfertilized egg has also been asserted by many other investigators apart from considerations of viscosity differences.

The vitelline membrane is probably not sharply delineated from the less viscous egg cytoplasm. According to Chambers ('17a) the external jellied surface of the egg cell "gradually passes into the sol of the interior." This gradation must be very abrupt as I did not notice it in my experiments although I think the superficial cytoplasm does undoubtedly adhere to the vitelline membrane.

3. *The Morphology of the Fertilization Process.*—The fertilization reaction has been observed on eggs mounted in plenty of water without a cover glass; on eggs in a depression slide with a cover glass; and on eggs in a hanging drop on the under surface of a cover glass placed over a depression. The reaction has been studied with low, medium, and high powers. The phenomena observed were the same by any method of mounting the eggs and cannot possibly be ascribed to compression, for the eggs were never under compression.

The fertilization reaction begins to be visible 45 to 60 seconds after mixing the eggs with dilute sperm suspension. It is first indicated by a roughening or crenation of the egg at one place on the surface and this roughening spreads very rapidly in all directions from the initial place over the entire surface of the egg. Following closely upon this change the vitelline membrane begins to elevate. This elevation starts at the same place as the roughening and like the latter sweeps in all directions over the egg. The roughening and the elevation of the membrane occur at such a close interval as to be almost simultaneous but it is certain that the former precedes. There is also to be noted a flattening of the egg at the region of initial membrane elevation. This flattening is commonly very marked at the site of initial membrane elevation and spreads from this for a short distance but never extends more than halfway over the egg. The fertilization changes in *S. franciscanus* are illustrated in Figs. 1 to 5.

The time required for these changes to pass over the egg is

about 15 to 30 seconds in the best eggs. In subnormal eggs the time is much longer and the reaction may not be complete, the eggs remaining permanently with membranes partially elevated as shown in Figs. 6 to 8. Such cases serve, I think, to indicate further the progressive character of the fertilization reaction.
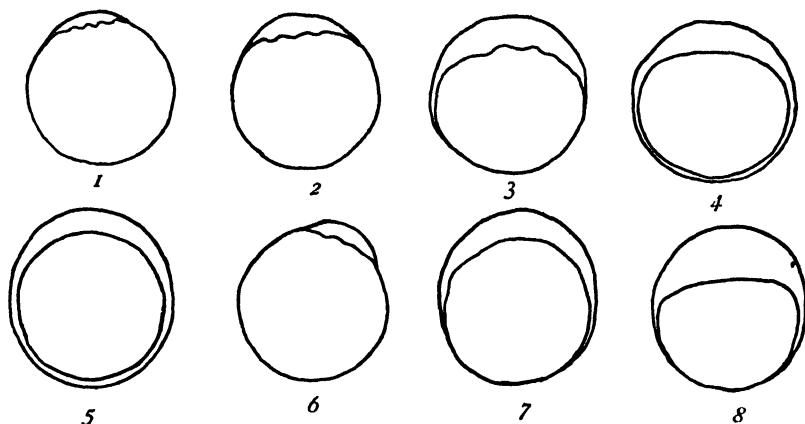
All figures are redrawn from free-hand sketches. In the unfertilized egg the vitelline membrane is not indicated separately from the egg surface as doing so would exaggerate the real appearance.

Figs. 1 to 5. Five stages in the normal fertilization reaction in *Strongylocentrotus franciscanus*. Note progress of the fertilization reaction from the initial place. In 5 the reaction is not yet quite complete as the egg is still slightly excentric within the membrane.

Figs. 6 to 8. Permanent stages of partial fertilization from a subnormal lot of eggs of *S. franciscanus.*

In eggs favorably placed for such observation it has been determined that the point on the egg from which the fertilization changes take their origin is the place to which the successful sperm is attached. This has also been ascertained by so many previous observers, to whom reference will be made shortly, that it seems superfluous to dwell upon the fact. The fertilization reaction begins at the point of contact of sperm and egg and from this point is transmitted in all directions over the surface of the egg.

The fertilization reaction is essentially the same in all three species studied. The chief difference between the urchin egg and the starfish egg is that in the former the membrane where it first separates from the egg elevates at once in this region to its

fullest extent whereas in the starfish egg the membrane separates from the entire surface of the egg before it elevates to any considerable extent (Fig. 9). Consequently in the urchins the egg at first lies very asymmetrically placed within the membrane (Figs. 4 and 5). This asymmetry is further emphasized by the flattening of the egg at the region of initial elevation. Later symmetry is restored in normal eggs by the widening of the perivitelline space at other regions and by the resumption of spherical form by the egg. In subnormal eggs the asymmetry is likely to persist. In the *Patiria* egg, the membrane separates from the whole surface of the egg with the formation of only a very narrow perivitelline space; later this widens simultaneously around the egg. The flattening at the region of initial membrane elevation is slight in the starfish egg but generally perceptible.
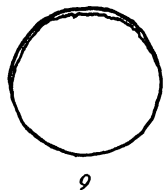
FIG. 9. Stage in the fertilization reaction of *Patiria miniata*. The vitelline membrane separates only slightly from the egg at first. Note also the crenation of the egg surface.

The separation of the vitelline membrane does not proceed with entire smoothness from the point of initiation but the membrane tends to adhere to the egg surface at some points slightly longer than at others. An exaggeration of this tendency results in the vesicle formation described and figured by Loeb ('13), observable according to him through retarding the normal reaction by lowering the temperature. Vesicle formation also occurs at ordinary temperatures in subnormal eggs and in such eggs the vesicles may persist, not flowing together to form a continuous perivitelline space. Pronounced vesicle formation seems to be due to a subnormal response of the egg to fertilization.

An attraction cone at the site of sperm entry such as has been reported by others was not noticed but was not particularly sought for. I was also unable to observe the passage of materials from the cortex into the perivitelline space, as described by Just ('19) for *Echinarachnius*, although I searched carefully for such a process.[1]

[1] In making this statement I do not intend to imply the slightest question of the validity of Just's observations. I believe from other lines of evidence that material does pass from the egg into the perivitelline space but the process is invisible in the eggs with which I have worked.

The progressive character of the fertilization reaction has been previously described by a number of investigators. It was first noticed about simultaneously by Fol ('77, '79) and Calberla ('78). They observed that the detachment and elevation of the fertilization membrane are initiated at the point of attachment of the successful sperm and proceed from there in all directions around the egg. Fol [1] described this process for *Asterias glacialis*, *Toxopneustes lividus*, and *Sphærechinus brevispinosus*. Calberla's investigations concern the *Petromyzon* egg. This egg possesses a micropyle; no change occurs until the sperm has passed the length of the micropyle and touched the surface of the egg cytoplasm inside the membrane. When this happens the egg withdraws a little from the vitelline membrane at the micropyle and flattens slightly; the separation of the membrane from the egg followed by its elevation then proceeds from the micropyle over the egg. Théel ('92) describes the elevation of the membrane in the sea-urchin *Echinocyamus* in the following words: "At the place where the first sperm has penetrated the mucilaginous investment, a very thin plasmatic membrane rises and separates from the egg, beginning at the place of contact and extending eventually around the yolk." Herbst ('93) agrees with Fol's description of the fertilization reaction in the sea-urchin egg. Ries ('09a) also observed the progressive character of membrane elevation in the sea-urchin egg and presents photographs of the process, taken with a motion picture machine. Elder's drawing of the elevation of the fertilization membrane in *Strongylocentrotus purpuratus* shows that this process is a progressive change initiated at one place but Elder does not mention this in the text ('12). Okkelberg ('14) has described the elevation of the membrane in the egg of the brook lamprey *Entosphenus wilderi*. In this egg fertilization occurs at the animal pole and the membrane here separates from the egg. A wave of contraction then passes over the egg towards the vegetative pole, separating the membrane from the egg. Just ('19) has given a careful description of the fertilization reaction in *Echinarachnius parma*. The reaction

[1] There seems to be a prevailing impression that Fol worked with compressed eggs. This is not, however, the case as Fol was at particular pains to state that the eggs were not in the least compressed ("sans les comprimer le moins du monde," '79, p. 176).

is similar to that in other echinoderm eggs. "The cortex reacts to penetration by pushing out a blister at the site of sperm entry." "From the point of sperm entry a definite gradient of membrane elevation is established, the last point of membrane elevation being at the pole opposite that of successful sperm entry."[1]

It therefore appears that in many eggs—echinoderms and lampreys—a change in the surface of the egg is initiated by contact of sperm and egg and that this change, which includes membrane separation and elevation, progresses from the point of contact in all directions to the opposite pole of the egg. It is probable that more careful observation would reveal the progressive character of the fertilization reaction in other eggs.[2] The wave-like progression of the reaction irresistibly suggests that electric phenomena are involved; recently Gray ('22) has made the same suggestion.

The roughening of the egg at fertilization has also been noted by a few observers. Schücking ('03) and Loeb ('13) record it for echinoderm eggs. The "peristaltic wave" which according to Okkelberg ('14) passes over the lamprey egg in normal fertilization or artificial activation is, I think, of the same character. Mr. Leigh Hoadley informs me that a roughening also occurs in the *Arbacia* egg on fertilization. The cause of the roughening is discussed later.

Flattening of the egg at the site of sperm entry has also not escaped observation and is regarded by some as a contraction. Hertwig ('78) probably has reference to this flattening when he states that in the starfish egg on fertilization "zieht sich der Dotter von der Eihaut zurück." Some of Fol's figures ('79) show this flattening and it is also recorded for *Toxopneustes* by Selenka ('78) and for *Petromyzon* by Calberla ('78). Schücking ('03) speaks of a contraction of the egg away from the vitelline membrane and probably has reference to the same phenomenon. The photographs of Ries ('09a) of fertilization in *Strongylocentrotus* plainly show the flattening at the site of initial membrane elevation and this is also mentioned by him in the text. Elder's

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[1] Professor F. R. Lillie informs me that while at Pacific Grove in the winter of 1920 both he and his assistant, Mr. J. Nelson Gowanlock, observed the progressive character of the fertilization reaction in *Strongylocentrotus*.

[2] But not in teleost eggs, according to Reighard '93.

drawing ('12, Fig. 6) of fertilization in *S. purpuratus* is similar. Gray ('16) speaks of the compression of the egg at fertilization by the contents of the perivitelline space.

Watching the fertilization process one certainly gains the impression that the flattening of the egg is due to a pressure exerted on the egg by the contents of the perivitelline space. It appears that the filling of this space does not occur *pari passu* with the elevation of the membrane but is due to some other process. This suggests that the accumulation of materials in the perivitelline space is the direct cause of the elevation of the membrane and such an idea has been advanced by many investigators.[1] Further evidence on this matter is desirable.

4. The Identity of the Vitelline Membrane with the Fertilization Membrane.—This matter has been the subject of some dispute in the history of the fertilization problem. Harvey ('10, '14), McClendon ('11), Elder ('12), Loeb ('13), and recently Gray ('22) have expressed the view that the fertilization membrane is formed by the precipitation or coagulation of materials emanating from the egg on contact with the jelly or the sea-water. That the jelly is not concerned in the appearance of the fertilization membrane has been shown by Harvey ('14) and Lillie ('14, p. 553).

On the other hand the identity of the fertilization membrane with the preëxisting vitelline membrane of the unfertilized egg has been maintained by many investigators: Fol ('77, '79) for *Asterias, Sphærechinus,* and *Toxopneustes,* Hertwig ('78) for *Asterias,* Calberla ('78) for *Petromyzon,* Théel ('92) for *Echinocyamus,* Reighard ('93) for teleost eggs, Herbst ('93, '04) for *Sphærechinus, Echinus,* and *Stronglyocentrotus,* Delage ('01) for *Asterias,* Schücking ('03) for *Asterias* and *Strongylocentrotus,* Ries ('09a) for *Strongylocentrotus,* Allyn ('12) for *Chætopterus,* Glaser ('13) for *Arbacia* and *Asterias,* Heilbrunn ('13, '15, '20b) for *Arbacia* and *Cumingia,* Okkelberg ('14) for *Entosphenus,* and Chambers ('21a, '21b) for *Arbacia, Asterias,* and *Echinarachnius.* Most of these authors rest their view on direct observation of

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[1] *E.g.,* Herbst '93, '04, Schücking '03, Ries '08, '09a, Loeb '08. It seems clear that the contents of the perivitelline space consist chiefly of water which enters from the outside through the vitelline membrane. This was proved for the lamprey egg by Calberla ('78) by coloring the water and is certain for teleost eggs (Reighard '93). The perivitelline space also appears to contain some colloidal material probably of protein nature derived from the egg.

the elevation of the vitelline membrane as the fertilization membrane. Such evidence is not entirely satisfactory for the echinoderm egg as the vitelline membrane is not clearly visible on such eggs. Chambers' evidence appears to be conclusive. He has shown that if the vitelline membrane be removed from unfertilized eggs (*Arbacia, Asterias, Echinarachnius*), such eggs do not elevate membranes on fertilization. Further by various methods the vitelline membrane can be made more obvious on the unfertilized eggs at certain points and the continuity of these easily visible portions of the membrane with the fertilization membrane after insemination is easily observable.

Further conclusive evidence of the identity of the vitelline and fertilization membrane is afforded by the study of the fertilization reaction in egg fragments. That portions of eggs may elevate fertilization membranes has been noted by Ziegler ('98), Moore ('12) and Glaser ('13). The fragments in these experiments were obtained by shaking the eggs and hence it is not known from which portions of the eggs they originate. It is certainly not justifiable to conclude from such experiments that any portion of the egg when isolated can reform a membrane capable of elevation since: (a) the cytoplasm being relatively fluid, much of it will be lost on rupture of the eggs and the cortical portion which adheres to the membrane will be most likely to persist; and (b) since it has been proved by Chambers ('21a, '21b) and Just ('23) that the interior of the egg is not fertilizable but only the cortex possesses this property, it follows that any fragments which fertilize must necessarily have retained a portion of the egg cortex and hence of the original vitelline membrane. It seems likely that most of the egg fragments obtained by shaking represent the whole cortex, the fluid interior having escaped.

I have studied the fertilization reaction in fragments of the eggs of all three species used. These fragments have been obtained by the method already described. An egg is held by surface tension and then punctured. The cytoplasm flows out and the egg begins to disintegrate. At various stages in this process the egg is rapidly pushed back' into the interior of the drop. The outflow at once ceases when the pressure of surface tension is removed and portions of eggs of all sizes and shapes are obtainable. Such fragments of course always retain a portion of the

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original cortex and vitelline membrane and it is definitely known to the observer which is the original and which the new surface of the fragment. In *Patiria* the whole vitelline membrane clings to the fragment. The fragments show little tendency to assume the spherical form.

Upon the addition of sperm all such fragments give evidence of the fertilization reaction. The first change noted is an alteration of tension in the fragment. This is evidenced by the disappearance of the wrinkles from the vitelline membrane of fragments of urchin eggs and by a change of shape. The fragments tend to assume a more spherical form. This tendency towards sphericity is very marked in the larger fragments as in Figs. 10 and 11. From these observations the conclusion can be drawn that the change of tension is confined to the original cortex, since when the amount of cortex remaining is small there is much less change in the shape of the fragment than when it is large. The next change that is noted is the roughening of the surface. This occurs *only on that surface of the fragment which was the original surface of the egg*. In the smaller fragments the visible fertilization change stops at this point. The membrane follows the crenations of the surface as shown in Figs. 17 and 20; consequently the crenations cannot be due to the formation of small vesicles as supposed by Loeb ('13). In the larger fragments the roughening of the surface is followed by the elevation of the fertilization membrane. The membrane elevates *only on that surface of the fragment which was the original surface of the egg* and which therefore bears a portion of the original vitelline membrane. In the *Patiria* egg where the whole vitelline membrane remains attached to the fragments, the membrane elevated on the fragment is perfectly continuous with the empty portions of the vitelline membrane as shown in Figs. 19 to 25. The elevation of the membrane on egg fragments is illustrated in Figs. 10 to 25.

The fertilization reaction in eggs with extra-ovates is to the same effect. Glaser ('13) records that if the egg of *Arbacia* is ruptured so that part of the contents flows out, a fertilization membrane appears on one sphere but not on the other. According to my observations on the same egg, it is the cortical sphere which elevates a membrane. Chambers ('21a, '21b) also finds that extra-ovates in the starfish are not fertilizable and do not
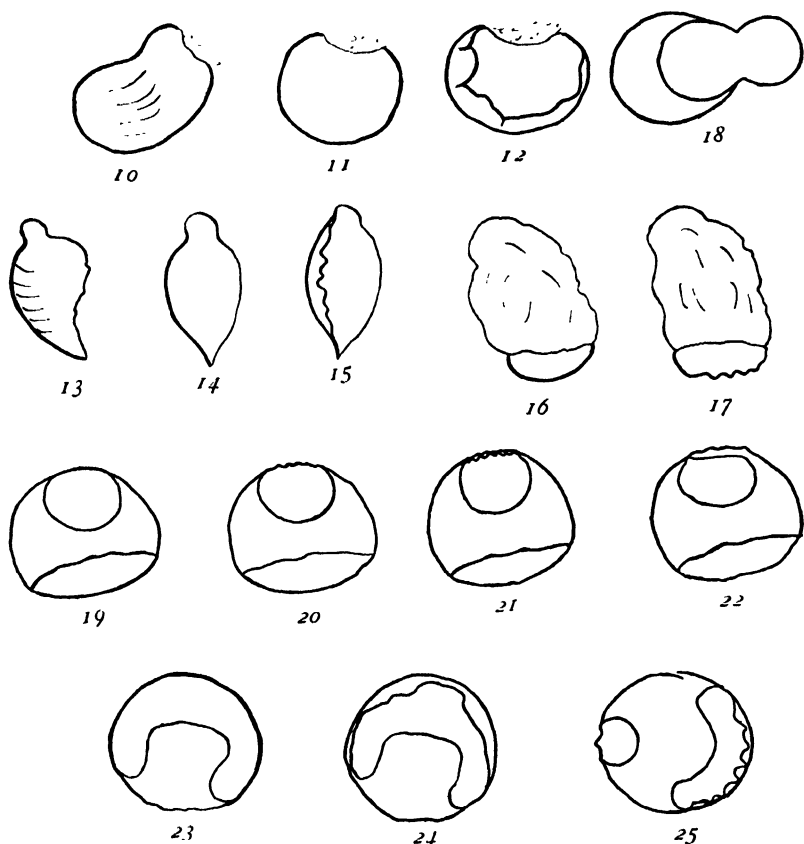
FIGS. 10 TO 25. Fertilization of egg fragments. The original surface is indicated by a heavier line. Figs. 10 to 18, fragments of *S. franciscanus*. Fig. 10, large egg fragment showing wrinkles in the vitelline membrane; Fig. 11, same after insemination; the fragment is rounded and the wrinkles have disappeared; Fig. 12, same later, with membrane elevated on the original surface. Fig. 13, smaller fragment; Fig. 14, same after insemination, showing change of form and disappearance of wrinkles; Fig. 15, same, later, with fertilization membrane elevated on original surface only. Fig. 16, small fragment with entire membrane attached; Fig. 17, same after fertilization; only the characteristic crenations appear; the membrane does not elevate on such small fragments. Fig. 18, extra-ovate of *S. franciscanus* after fertilization; cortical sphere to left, endoplasmic sphere to right; only the former elevates a membrane.

FIGS. 19 TO 25. Fertilization of egg fragments of *Patiria miniata*. Fig. 19, small fragment with entire vitelline membrane; Fig. 20, same after fertilization, showing the characteristic crenations; Figs. 21 and 22, same, later, showing separation of the fertilization membrane; it is continuous with the vitelline membrane. Fig. 23, large fragment of *Patiria;* Fig. 24, same after fertilization. Fig. 25, large and small fragment left within the same vitelline membrane, shown after fertilization; the small fragment exhibits only the characteristic crenations; the large one has elevated a fertilization membrane.

elevate fertilization membranes but that these processes are confined to the cortical portion of the ruptured egg. He further states that if the extra-ovate remains in continuity with the cortical portion for some time it becomes fertilizable but still does not elevate a membrane. I have produced extra-ovates in *S. franciscanus* with the needle; upon fertilization the cortical portion elevates a membrane while the extra-ovate does not (Fig. 18).

From all of these observations it may be concluded: (*a*) that the vitelline membrane is identical with and is elevated as the fertilization membrane; (*b*) that the vitelline membrane is a definite morphological structure which cannot be reformed or replaced;[1] and (*c*) that the fertilization reaction, which includes other visible changes besides membrane elevation, is exhibited only by the cortex of the egg.[2]

Since the fertilization membrane is thus a preformed, pre-existent structure, its separation from the egg at fertilization should be spoken of as membrane elevation and not as membrane formation. The advisability of this terminology was previously emphasized by Heilbrunn ('15).

When the vitelline membrane elevates as the fertilization membrane, however, it unquestionably undergoes certain physical and chemical changes. In the first place it would seem that the membrane must soften since in elevating it is distended considerably beyond its former circumference. A number of observers have attested that there is no decrease in size of the echinoderm egg at fertilization (Fol '79, Théel '92, Herbst '93, Schücking '03, Ries '08, Loeb '08, McClendon '10, Chevroton and Vlès '11, Gray '16, Chambers '21*b*). I was also unable to find any change in size of the eggs of *S. franciscanus* on fertilization.

[1] Any portion of the egg cytoplasm does of course when exposed to sea-water form a protective surface layer which has no doubt physiological properties similar to those of the vitelline membrane but which is not elevatable by sperm. Several observers (Herbst, '03, Tennent and Hogue, '06, Harvey, '10, Heilbrunn, '20*b*) have recorded that a second membrane can be elevated after the elevation of the usual fertilization membrane by treatment with various agents (but not with sperm). This second membrane is in reality the hyaline layer, as also stated by Harvey ('14) and Heilbrunn ('20*b*).

[2] The importance of the cortex finds explanation on the basis of Lillie's fertilizin theory (cf. Lillie, '19, also Chambers, '21*b*). Just ('23) also shows that the fertilization reaction is confined to the cortex.

Consequently the vitelline membrane is distended at fertilization, and such distension indicates a softening or degelation of the membrane. According to R. S. Lillie ('15) the temperature coefficient of heat activation in the starfish egg indicates that a degelation process is involved. Just's ('22) recent experiments show that the membrane [1] becomes very much less resistant at the places where it is separating from the egg for such places burst when the egg is exposed to diluted sea-water during elevation. Thus the decreased resistance or softening of the vitelline membrane like other fertilization changes is initiated at the point of contact of sperm and egg and is transmitted from this point over the egg. Within a few minutes after elevation the membrane appears to toughen again. This hardening or toughening of the membrane after elevation has been spoken of by several observers. Thus Herbst ('93), Goldfarb ('13), C. R. Moore ('16), F. R. Lillie ('21) have recorded that the fertilization membrane of urchin eggs is much more easily removed by shaking within a few minutes after fertilization than later. Chambers ('21b) also finds that the fertilization membranes of *Asterias*, *Arbacia*, and *Echinarachnius* begin to toughen very soon after they are elevated. The extensive hardening of the fertilization membrane in nematode eggs is well known. That the membrane also undergoes chemical alteration on elevation may be inferred from an experiment of Harvey's ('10). He found that the vitelline membrane is soluble in concentrated sulphuric acid while the fertilization membrane is not.

5. Viscosity Changes at Fertilization.—It was pointed out in the first part of this paper that the cytoplasm of the unfertilized egg is a slightly viscous fluid. It can be determined by the same procedure as described there that at fertilization there is a sudden and marked increase in viscosity. In the eggs of *Strongylocentrotus* (both species) this increase in viscosity occurs just before the membrane begins to elevate; this is probably also true of the *Patiria* egg although it was not determined with certainty. It is present in this egg at least as soon as the membrane lifts at the site of sperm entry. The viscosity increase is

[1] Just speaks only of the softening of the cortex but probably includes the membrane in this term. It seems evident that the membrane must also be involved in the softening otherwise it would not yield at the places in question.

well marked and occurs very suddenly, at the moment before membrane elevation is initiated. At this moment, the cytoplasm flows less readily than before and cuts and gashes made with the needle close up more slowly. The viscosity increases during the elevation of the membrane but appears to reach a maximum in a very short time.

We may therefore speak of an increased viscosity or gelation of the egg cytoplasm as one of the changes included in the fertilization reaction. I believe this gelation to be responsible for the roughening of the egg surface at fertilization. As Rhumbler ('05) has recognized, a rough and crenated surface is causally related to a solidified condition of protoplasm. As the roughening of the egg begins at the point of contact of egg and sperm and spreads from there over the egg, it follows that the gelation process must likewise originate at the site of sperm entry and progress from this point in all directions. It seems probable that the gelation process is confined to the cortex of the egg since in egg fragments only the original surface roughens at fertilization.

The interpretation of the rounding up of egg fragments remains to be considered.[1] I at first thought this to be another indication of increased viscosity, of the increased tension accompanying the change from a more fluid to a more viscous state. But obviously gelation cannot cause both a rounding up and a roughening. Although the surface tension of gels is higher than that of sols, still surface tension is not great enough to induce sphericity in fragments of gels. It therefore seems necessary to conclude that the rounding of egg fragments indicates a decreased viscosity or increased fluidity. As my observations show that this process precedes the roughening by a quite perceptible time interval, it seems that the cortex of the egg at fertilization first becomes more fluid and then undergoes gelation. Just ('22) emphasizes a liquefaction of the cortex as part of the fertilization reaction.

The gelation of the egg at fertilization serves at least two purposes: (a) the vitelline membrane is split from the egg cyto-

[1] Harvey ('10) has also emphasized the rounding up of eggs at fertilization and attributes it to an increase in tension. However, the same tension will induce sphericity if the cytoplasm becomes more fluid. My observations on egg fragments indicate that this change in tension is in the cortex, not in the membrane.

plasm and acquires a definite internal boundary which it had hitherto lacked;[1] and (b) a new surface is formed on the egg cytoplasm, the so-called hyaline layer, which prevents the egg contents from expanding with the membrane and which replaces physiologically the vitelline membrane. The separation of the vitelline membrane from the egg cytoplasm at fertilization thus appears to me to be a process quite independent of the subsequent elevation of the membrane. The hyaline layer which replaces the vitelline membrane requires some time for its complete development and is according to Chambers ('21b) "firm and gelatinous."

An increased viscosity accompanying the fertilization reaction has not been hitherto recorded. Several observers have, however, noted such an increase following after fertilization, recently Heilbrunn ('15, '20a, '21), Chambers ('17b, '19), and Seifriz ('20). According to these investigators the increased viscosity is associated with some phase of the mitotic figure. According to Chambers ('17b) the gelation after fertilization is at first limited to the small sperm-aster and later spreads throughout the egg. While fully accepting the conclusion of these authors that asters and spindles are gelation figures I do not think that the initial gelation which constitutes part of the fertilization reaction is due to the sperm aster. The latter is at first localized around the sperm head while the gelation which I am considering appears to be general throughout the whole cortical region of the egg.

Since the viscosity changes at fertilization precede the elevation of the membrane it may be emphasized that the latter is a secondary rather than a primary phenomenon in the fertilization reaction. It seems certain that changes have taken place in the egg before the membrane elevates. This has recently also been emphasized by Just ('19): "In the *Echinarachnius* egg, normal development has already been initiated by the sperm when the membrane begins to form." It appears that the elevation of the membrane is not due directly to sperm penetration but is the result of changes in the egg.

6. Artificial Membrane Elevation and Cytolysis.—It is well

[1] Fol ('79) in particular emphasizes that the vitelline membrane (couche enveloppante) lacks a definite internal boundary and that it acquires one at fertilization.

known that a number of chemical substances as well as other agents will induce a fertilization membrane in echinoderm eggs. This matter has been discussed from the point of view of membrane formation by Traube ('09), Loeb ('13), Gray ('22) and others and from the point of view of membrane elevation by Heilbrunn ('13, '15). I have made a few observations on artificial membrane elevation, using chiefly ether and diluted sea-water or distilled water but also butyric acid. In employing ether and similar substances for membrane elevation, the eggs must be rapidly returned to normal sea-water, as noted by Loeb ('13) if any are to be saved from cytolysis.

After the application of membrane-elevating substances such as ether and distilled water, three classes of eggs are noted: those with blister-like elevations, those with completely elevated membranes, and those which are cytolyzed. In a considerable number of eggs after treatment with ether and distilled water, the membrane is elevated only as local blisters. In one or two cases such blisters were observed to spread over the egg until the entire membrane was elevated but generally they persist unchanged as long as observed (unless cytolysis intervenes). They appear to be due to chance inequalities of contact with the membrane-elevating solution when the latter is first applied and indicate that the local action of such solutions is incapable of inducing complete membrane elevation. Such partially elevated membranes can be completed by sperm. The portions elevated by sperm appear to be continuous with those elevated by the agent.

After treatment with ether and distilled water there is obtained a small percentage of eggs in which the membrane is completely elevated and which cannot be distinguished visibly from eggs fertilized by sperm. Investigation with the needle shows, however, that as concerns viscosity conditions these eggs are entirely different from normally fertilized eggs. Whereas in the latter, as already noted, the cytoplasm has undergone gelation, in these eggs with artificially elevated membranes there is no trace of such increased viscosity. These latter eggs are as fluid as or more fluid than normal unfertilized eggs. This agrees with Heilbrunn's ('20c) statement that ether, chloroform, and similar

substances liquefy the *Arbacia* egg. It is thus evident that the response to membrane-elevating substances, so far as tested, is not equivalent to the response to sperm.

The usual butyric acid treatment yields, after return to normal sea-water, a percentage of eggs with membranes of normal appearance equal to that produced by sperm. But in such eggs, also, the normal gelation appears to be lacking.

The majority of the eggs treated with membrane-elevating substances such as ether and distilled water undergo a change designated by Loeb as cytolysis.[1] This condition is sufficiently described in Loeb's book ('13). The eggs are much expanded and transparent. It appears from Loeb's description ('13, p. 188) that he regards this cytolysis to consist in an absorption of fluid followed by a liquefaction of some of the egg contents. It can readily be shown by the needle that this conception of cytolysis is erroneous. Cytolysis is not a liquefaction of the egg contents; it is a complete and irreversible coagulation. This fact was discovered by Heilbrunn ('15) and the increased viscosity of cytolyzed eggs was also noted by Goldfarb ('18). When cytolyzed eggs are punctured with a needle, a small amount of watery fluid generally escapes (this is probably water which passes in from the outside when the membrane expands) but the egg material itself will no longer flow. It is completely solidified and can be cut into pieces with a needle.

The coagulation [2] caused by membrane-elevating solutions is entirely different from the normal gelation attendant on fertilization. The cytolytic change is an irreversible lethal change in which the egg colloids are precipitated out in a coagulated mass. The viscosity of the cytolyzed egg is very much greater than that of the normally fertilized egg at any time from fertilization to the first cleavage. The normal gelation on the other hand is a reversible physiological process in which there is no such precipitation of colloids.

The cause of cytolysis appears to be as follows. Since after

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[1] My remarks refer only to the "white" cytolysis of Loeb.

[2] The term coagulation can of course be used to designate any marked increase in viscosity. It seems preferable to me, however, to confine the term to an irreversible separation out of colloids and to use the term gelation to designate reversible physiological increases in viscosity.

treatment with membrane-elevating solutions without cytolysis, the membrane elevates without an accompanying cytoplasmic gelation, it is evident that the egg is left without a resistant surface. Consequently the surface of the egg after lifting of the vitelline membrane is weak. The membrane-elevating substances tend to induce the expansion of the egg in the same way as they induce the expansion of the membrane (cf. further Heilbrunn, '15) and as the egg is not protected by a resistant surface it naturally ruptures by expansion. Such rupture causes coagulation since it has been shown by the microdissectionists (Chambers, '17a, Seifriz, '20) that injury leads to coagulation. The agents in question do not cause coagulation directly but act by elevating the protective vitelline membrane from the egg, leaving the cytoplasm without a sufficiently resistant surface.

From these considerations it is highly questionable whether the cytolytic action of parthenogenetic agents has any relation whatever to their parthenogenetic power or whether any conclusions can be drawn concerning the mechanism of normal activation from the cytolytic properties of such agents. A similar conclusion as to the lack of relation between cytolysis and activation has been reached by Just ('20) from other lines of evidence. It seems sufficiently evident that cytolysis is simply a death change and has no bearing on activation.

It occurred to me to determine whether artificial agents can elevate membranes on eggs in which the sperm are not able to do so. It is well known that after standing in sea-water for twenty-four hours or more urchin eggs no longer elevate membranes on fertilization although they are still capable of development. The vitelline membrane probably loses its elasticity and capacity for distension after a prolonged stay in sea-water. I found a considerable degree of parallellism between the action of sperm and of artificial agents on such eggs. It is much more difficult and in some cases impossible to induce membrane elevation by agents in eggs which do not elevate membranes on insemination. In most cases, however, the artificial agents are more or less effective in partially or completely elevating the membrane. The action of these agents is thus more powerful than that of the sperm and may possibly be of a different nature.

In some cases membrane elevation could not be induced by arti-
ficial agents in these stale eggs.   In such cases the egg bursts
through the membrane, forming either a number of vesicles or
erupts after the manner of an extra-ovate.   This further indicates
that the agents used have an expansive effect upon the egg con-
tents as well as on the membrane.   When extra-ovates are formed
from stale eggs by these agents the vitelline membrane separates
from the egg contents.   As this is not the case when extra-ovates
are induced in fresh eggs, one may again conclude that the vitel-
line membrane loses its elasticity and distensibility on standing.
The membrane appears much stiffer and firmer than in fresh
eggs; in all probability it is coagulated.

   7. *Conclusion.*—In conclusion I may be permitted to reiterate
an old view that the activation of the egg by the sperm is of the
nature of a stimulation or excitation.   Among the characteristics
of a stimulation are: (*a*) many different agents are capable of
exciting the same effect in the protoplasm which is stimulated
so that there is no specific relation between the properties of the
agents and the change invoked in the protoplasm; (*b*) the changes
induced in the protoplasm stimulated are altogether in excess of
the energy content of the stimulus; (*c*) the changes invoked in
the stimulated protoplasm depend upon the constitution of the
protoplasm and not upon the nature of the stimulus; *d*) the
excitation is transmitted from the point of application of the
stimulus.   It is evident that the activation of the egg exhibits
these characteristics.   Many different agents are able to induce
activation.   There appears to be no specific relation between the
changes induced in the egg and the physical and chemical proper-
ties of the stimulating agents.   To suppose that the sperm brings
into the egg some substance which evokes the activation changes
is I think just as far from the truth as to suppose that the various
agents which induce a nerve impulse do so by injecting some
substance into the nerve.   I think one must agree fully with
Lillie ('19, Chap. VII) that the egg is "an independently activ-
able system" and "possesses all of the substances necessary for
activation."   Finally it has been shown in at least a number of
cases that the fertilization reaction is initiated at the point of
sperm entry and is transmitted from this place over the egg.   The

transmitted changes recorded in this paper are: the roughening (gelation) of the egg and the elevation of the fertilization membrane. To these may be added those recorded by Just ('19, '22)—the loss by the egg of fertilizibility, the passage of materials from the cortex into the perivitelline space, and the softening of the vitelline membrane.

The problem of the activation of the egg becomes thus a problem of the nature of stimulation in general and cannot be solved until the more general problem has attained solution.

8. *Summary.*—(*a*) The eggs used were those of *Strongylocentrotus franciscanus* and *purpuratus* and *Patiria miniata*.

(*b*) Physically these eggs consist of a slightly viscous cytoplasm inclosed in a vitelline membrane of solid consistency.

(*c*) In all three species the fertilization reaction begins at the point of attachment of the successful sperm and is transmitted from this place in all directions over the egg.

(*d*) The visible manifestations of the fertilization reaction are a roughening of the surface and the elevation of the vitelline membrane. Both begin at the site of sperm entry and spread from there over the egg.

(*e*) The vitelline membrane is identical with and is elevated as the fertilization membrane.

(*f*) In fragments of eggs only that surface of the fragment which was part of the original surface of the egg shows the fertilization reaction-roughening and membrane elevation.

(*g*) The vitelline membrane cannot be replaced or reformed.

(*h*) At the moment of fertilization just preceding the elevation of the vitelline membrane an increased viscosity or gelation occurs in the egg. This splits the vitelline membrane from the egg cytoplasm and provides a new resistant surface for the latter. This gelation is the cause of the roughening of the egg at fertilization.

(*i*) A change in the egg thus precedes membrane elevation and makes it probable that the latter process is not the primary event in the fertilization reaction.

(*j*) After membrane elevation by such artificial agents as were tested there is no increased viscosity in the egg but the cytoplasm is of the same or of less viscosity than the unfertilized egg, until cytolysis occurs.

(*k*) Cytolysis consists of an irreversible coagulation of the egg cytoplasm and appears to be due to the fact that the egg after the action of cytolytic agents is left without a resistant surface. It consequently ruptures and this injury leads to coagulation. Probably cytolysis bears no relation to activation.

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BIOLOGICAL BULLETIN

THE ORIGIN OF THE MYCETOCYTES IN PSEUDOCOCCUS.

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INTRODUCTION.

Symbiotic fungi or mycetozoa which are to be found in many species of insects, have lately been made the subject of some interesting work chiefly in Germany and Italy. This peculiar association between fungus and insect body seems especially well developed in the Homoptera and there a great variety of special features is to be found in its development. In some cases, among which are the mealy bugs of the genus Pseudococcus, the symbionts are lodged in or associated with cells that originate in the insect body—the mycetocytes. These cells have a peculiar interest in that they are evidently very much specialized and are restricted to a very definite locality in the body of the insect. In all of the Homoptera, the symbionts are transmitted from one generation to the next through the eggs; each of which receives a certain number of the fungi from the mycetome or symbiont mass of the mother. In *Pseudococcus*, such a transfer to the ova involves a dissociation of the symbionts from the mycetocytes, for the latter do not pass into the eggs. When the infection of the egg is complete, the fungi are therefore found "naked" near the anterior pole and always in the form of a number of spherical clumps or packets, each of which contains a large number of the symbionts. During the development of the embryo, these clumps once more become associated with mycetocytes which arise in some way in the embryo.

The exact nature of the mycetocytes has received a variety of interpretations. Breest ('14), working on *Aspidiotus*, a coccid

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not distantly related to *Pseudococcus*, suggested that they arise from the yolk cells—that is, cells that remain behind in the yolk at the time of blastoderm formation.   ·

Strindberg ('19), who worked on *Lecanium*, reported that in that coccid also, the mycetocytes took their origin in the yolk cells.

Pierantoni, who was the first worker to make a detailed investigation of *Pseudococcus* gives a different account of the mycetocytes ('10, '11, and '13). According to him, some of the cleavage cells in traveling to the periphery to establish the blastoderm, encounter the symbionts. This association, more or less accidentally initiated, becomes permanent, and the cleavage cells assume the characters that stamp them as mycetocytes. Exactly the same process has been described by Pierantoni also in *Icerya*.

Buchner ('21) in reviewing previous investigations, seems inclined to agree with Pierantoni.

Finally, Shinji ('19), also working on *Pseudococcus*, describes a migration of cells to the symbionts, shortly before the germ band commences its growth. These migrating cells he interprets as potential germ cells. Some of them become permanently associated with the symbionts and constitute the mycetocytes, but others migrate once more to form the definitive gonads.

In criticizing these varying results, I am not in a position to pass judgment on the conclusions of Breest and Strindberg. Certainly my own results in regard to the point in question, *i.e.*, the origin of mycetocytes, have led me to an entirely different interpretation.[1] But this difference may very well be due to an actual difference in the development of mycetocytes in the three genera under consideration.

The work of the other investigators mentioned has already been considered in a recent paper ('23). Although a considerable part of that paper was devoted to showing that Shinji's conclusions are untenable, I also took up briefly the statements of Pierantoni. My own position on this question of the nature of the mycetocytes can best be presented by giving a short resumé of those of my findings that are involved in the present discussion.

In both *Pseudococcus citri* and *P. maritimus*, the somatic num-

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[1] Regarding other points in the early development, I am in essential agreement with Strindberg.

ber of chromosomes is ten, all being alike in size and shape (Figs.
1–3).   Five tetrads are formed in the maturing egg, and the egg
nucleus undergoes a reduction and an equation division.   The
order in which these two divisions occur cannot be ascertained.
The first division results in two daughter groups each consisting
of five dyads.   One of these groups of course represents the first
polar body.   It is not extruded but remains in an inactive state
at the periphery.   The other group of dyads undergoes the second
maturation division, the results of which are two groups, each
containing five unit chromosomes or monads.   One of these last
two groups remains at the periphery, constituting the second
polar body.   The other sinks into the egg and there combines
with the male pronucleus.

The five dyads of the first polar body now break up into their
unit elements, ten in number, and then enter the resting condi-
tion.   The five monads of the second polar body also become
diffuse.   The two polar bodies then approach until in touch
with each other.   Fusion may then actually occur and I have
described such a case in my previous paper ('23).   In other cases
however fusion is delayed until the chromosomes of each have
been almost completely reformed and the nuclear walls begin to
break down.   The chromosomes then intermingle to form a
single group, and this of course contains 15 chromsomes.   With
this act of combination the polar nucleus becomes established
(Fig. 4).

The polar nucleus undergoes two or three divisions which
appear normal in every respect (Fig. 4 to 7).   Following the last
of these divisions, irregularities occur, and in the course of these
the chromosomes are greatly increased in number and the size
of the cells is enlarged.   These phenomena occur in every egg and
the resultant cells I have called giant cells.   The giant cells once
established, undergo some apparently normal divisions and
then become separated from the periphery and migrate a con-
siderable distance through the egg to the symbionts.   With these
they enter into association and thus form the mycetocytes, which
in this manner are formed anew in every embryo.

Although this account portrays the general course of events,
it leaves unexplained the exact nature of the irregularities which

convert the derivatives of the polar nucleus to giant cells. It is this point with which the present paper is concerned.

The work was done on two species, *Pseudococcus citri* and *P. maritimus*.

CHROMOSOME COUNTS.

The short statement regarding the origin of giant cells was given as follows in my previous paper ('23): "Later divisions of the polar nucleus derivatives are subject to irregularities. Apparently nuclear division is then very often or even generally not accompained by cytoplasmic division, so that the two resulting nuclei may lie side by side in a single protoplasmic area. At the ensuing division, there may be an intermingling of the chromosomes evolved, or else a multiplicity of spindles. Possibly also, cleavage cells nearing the edge may at times fuse with the derivatives."

This statement covers the problem only in a very general way and is hardly definite enough to be regarded as a solution of the complexities that are to be observed in the behavior of these peculiar cells. The one point established is that the polar nucleus derivatives are involved in some way in giving rise to the giant cells.

Without making any reference to this early period in the embryology of *Pseudococcus*, Buchner ('21) in his work on symbiosis comments on the fact that the mycetocytes in the adult contain a multiple number of chromosomes. This condition I had also observed, but it was not until recently, when the embryology was worked out, that the relationship between the giant cells of the embryo and mycetocytes in the adult became clear to me. Buchner apparently did not study the younger stages of *Pseudococcus* and therefore did not observe the giant cells at all.

The giant cells when first making their appearance in the egg are marked by a peculiarity that was observed very early in my investigations. This is, that although the number of chromsomes they contain is clearly variable and always greater than the true somatic number, it nevertheless varies only within very definite limits. This led to a more careful examination of the chromosome numbers in these cells.

A large number of chromosome plates was investigated. Un-

fortunately, the large size of the cells and plates often results in their being cut in the process of sectioning. Even if the chromosome plate studied is entire and flat, the chance of an overlapping of several chromosmes is very great, considering the large number involved. The latter defect may often make a count unreliable, but the cutting of a plate must throw it out of consideration at once. Discarding therefore all counts in which any doubt as to the number can possibly be entertained, the following data were obtained:

There are 6 plates containing 25 chromosomes; 3 plates containing 30 chromosomes; and 5 plates containing 35 chromosomes. These numbers become more impressive in view of the fact that during this period not a single perfect plate carrying any other but these three numbers was encountered (Figs. 12–17).

Among the plates discarded because overlapping of chromosomes made their counting uncertain, there were several in which doubt existed only with respect to a single chromsome, *i.e.*, whether to count it as one or two. Accordingly as these two possibilities were taken, these doubtful plates were found to fall under one of the three types given, or differ from it by one chromosome. In this uncertain group of plates there are 5 plates containing 25, or 1 more or less chromosomes; 3 plates containing 30, or 1 more or less chromsomes; 1 plate containing 35 or 36 chromosomes. Again, no plate in which doubt as to one chrom-. some exists was found to approximate any number but 25, 30 and 35.

How are these numbers to be explained?

In every perfectly clear plate, it was evident that the chromosomes resemble each other very closely as to size and shape. This fact coupled with the observation that the multiple numbers found are all multiples of five makes it improbable that an irregular process of fractionation of the chromosomes is to be held accountable. Again, although a definite regularity obtains in these chromsome numbers, it has also been observed that one egg may carry cells of more than one type. Thus a plate with 25 may be found close to another plate with 35 chromosomes. This too would be difficult to explain on the basis of a fractionation, especially as the character of the numbers would make

inevitable the hypothesis that only certain definite chromosomes break up into smaller units.

If effective polyspermy were common, the possibility that the supernumary sperms might combine with each other or any of the embryonic cells should receive some consideration. It would then remain to be explained why such numbers as 20 or 40 should not be encountered, since the 5 chromosomes of each sperm should make almost any combination possible. But aside from this consideration, I may point out as I have previously done, that supernumary sperms even if present rarely evolve chromosomes and certainly not as part of a regulated process, whereas giant cells with their multiple numbers of chromosomes are to be found in every developing egg at the right stage.

Finally, as the last of these possible but unlikely hypotheses remains an irregular behavior of the polar bodies. It is perfectly possible that the first and second polar bodies may not combine and that one or both may continue to divide independently. In combination with embryonic cells, the second polar body might thus bring about such numbers as 25 and 35. But against this I need repeat only my former observations that in all eggs at the crucial stage under observation, the polar nucleus is always formed and its 15 chromosomes go through several normal mitoses. None at all show an independent development of the two polar bodies. This of course still leaves the possibility that the first polar body may at times divide before fusion with the second. It may even be admitted that independent divisions or development of the polar bodies may sometimes occur. But such cases, and I have not seen any, are not normal.

These considerations leave only two types of cells as factors in the origin of the giant cells. They are the polar nucleus derivatives carrying 15 chromosomes and the true cleavage cells with 10 chromosomes.

Taking up the three types of giant cells in order, it is plain that the 25 chromosome type can arise only by a combination of a polar nucleus derivative (15 chromosomes) with one cleavage cell (10 chromosomes).

The 30 chromosome type must arise from a fusion, or recombination after mitotic division, of 2 polar nucleus derivatives.

Numerically, the same number could be attained by the combination of 3 cleavage cells. The latter possibility is more than doubtful if it is considered that giant cells are formed only in a certain and limited area at the periphery, whereas if a fusion of cleavage cells alone is a possibility such cells with 30 chromosomes should then be formed at any part of the periphery where cleavage cells are forming the blastoderm. Weighty although negative evidence against this hypothesis is to be adduced from the seeming nonexistence of giant cells with 20 chromosomes. The failure to find such cells is all the more significant if it is considered that the chances of two cleavage cells coming together for fusion are greater than the chances of three combining in that way.

The 35 chromosome type can originate only from a combination of a polar nucleus derivative with two cleavage cells. Other chances of combination to bring about this type have been ruled out by the preceding considerations.

Why a 40 chromosome cell should not be found at this stage it is not possible to say. Possibly there are such cells but their less frequent occurrence has prevented their discovery. More probably the three types already mentioned represent all the combinations possible.

The Giant Cell Chromosomes at Later Stages.

As already mentioned, the giant cells originate always at the time that the cleavage cells are migrating to the periphery of the egg to establish the blastoderm. With the completion of the latter and the first stages of germ band formation, another period in the history of the giant cells is initiated. In the course of this, giant cells with still greater numbers of chromosomes than those already described, are encountered. Beside them, the three older numerical types may continue to exist. The increase in the number of chromosomes, together with the fact that divisions in the giant cells tend to decrease their size, make certain counts in these later cells a much more difficult matter. Apparently most of these greater chromosome numbers hover in the neighborhood of 60 or 70. Only one certain count could be made, that being of a plate containing 60 chromosomes (Fig. 18).

Cytological evidence to be considered later, makes it probable

that the period of fusion has now been passed. It is indeed possible that there is an occasional combination of a giant cell with one of the yolk cells, the latter representing nothing but cleavage cells that failed to migrate to the periphery to establish the blastoderm. Fusion with what were formerly cleavage cells but must now be termed blastoderm cells, can of course take place only at the edge of the giant cell area, where the two types of cells are in contact. But aside from the fact that the giant cells in that location are not larger as a rule than those more centrally placed, it must be considered that the blastoderm cells in their expansion seem to exert actual pressure on the giant cells. The latter are heaped up and finally actually leave the periphery altogether. If such a pressure really exists, it would be constantly cancelled by fusion of adjoining giant and blastoderm cells.'

Nor can there be a continued tendency of giant cells to fuse with each other. Such occurrences are at least not general, for the number of giant cells is at this time slowly but steadily increasing, while their individual size is decreasing. Nevertheless the size is evidently variable, so that an occasional division of the chromosomes unaccompanied by cytoplasmic division remains as the most plausible explanation.

Once the giant cells have migrated to the symbionts and entered into association with them, divisions become rarer. At the same time it must be observed that in the adult *Pseudococcus*, the former giant cells, then called mycetocytes, contain relatively enormous numbers of chromosomes. The association with the symbionts must therefore have a disturbing effect on the few divisions that still occur, and most probably it is the failure of cytoplasmic division following a normal division of the chromosomes that thus causes a multiplication of the chromosomes. This idea has already been expressed by Buchner ('21).

CYTOLOGICAL EVIDENCE.

A complete cytological consideration·of the problem should begin with a study of the maturation phenomena in the egg. Since these primary steps have already been considered at some length in my previous paper ('23), it is sufficient to begin the present account with the polar nucleus, *i.e.*, the combined first

and second polar bodies. The first difficulty arises in determining the number of divisions to which the polar nucleus is subjected. It seems certain that at least two divisions take place regularly and that they are always normal. In some cases, the 4 nuclei or polar nucleus derivatives resulting from these two divisions certainly undergo a third division. But whether this last division occurs in every egg is not so certain. If so, there will then be 8 polar nucleus derivatives at the periphery of the egg (Fig. 4–7a).

Almost the same difficulties are encountered in determining the number of divisions that the fertilization or zygote nucleus undergoes, before the resultant cleavage cells take up their migration to the periphery to establish the blastoderm. Typically there appear to be about 32 cells in the interior of the egg when the migration begins.

It will be apparent that there is a distinct variation in the rate of division of polar nucleus derivatives and cleavage cells respectively. While the polar nucleus is undergoing at most 3 divisions, the fertilization nucleus undergoes approximately 5. The result of this is that while all the nuclei of each type taken by itself are at about the same stage of division, they may not be at all synchronous with the division stages of the other type. It is this condition that lies at the bottom of the difficulty in determining the number of divisions that the nuclei in question undergo before the process of fusion is begun. Thus in one egg, there are 8 polar nucleus derivatives, all in slightly varying stages of telophase, and still connected in pairs by spindle fibers (Fig. 7). The cleavage cells of this egg have begun the peripheral migration, but none have yet reached the edge. It can be assumed that here 8 polar nucleus derivatives will be involved in the processes of fusion to follow. In contrast with this is another egg in which there are only 4 polar nucleus derivatives. The chromosomes are in the final stage of condensation but the nuclear walls have not yet been broken down. The cleavage cells, present in about the same number as in the previously mentioned egg (32), are again in the stage of migration, and one has actually come in touch with one of the polar nucleus derivatives at the periphery. This cleavage cell like its sister cells is in the resting phase. The question therefore arises whether the polar nucleus derivative will complete its

impending division regardless of the proximity of the cleavage cell, or whether the presence of the latter will make that division abortive. In the first case, 8 nuclei will commence the fusion process as before; in the last named eventuality only 4 will be at hand (Fig. 7a).

On the whole it may be assumed that the polar nucleus as well as the fertilization nucleus undergoes a definite number of divisions. As has been noted previously, the chromosomes of any single giant cell are from their first appearance alike in size and shape. The chromosomes of the polar nucleus derivatives however decrease in size with each succeeding division (Fig. 4 to 6) and the same is true of the cleavage cells. If fusion or combination of these two types of cells could occur after a varying number of divisions, it would be expected that the chromosomes of the combination nucleus would often be of two sizes. But this, as has been said, is not the case. It is of course possible that there is some regulative mechanism capable of equalizing differing sizes of chromsomes, but for this assumption there is little or no basis.

Regarding the phenomena of fusion which now occur, the conclusions based on the numerical data receive the full support of the purely cytological evidence. In giving this last named proof I am fully aware of the ease with which in a case like the present, a number of isolated figures can be seriated to fit a preconceived hypothesis. Standing alone, the cytological proof would therefore be advanced with considerable caution. Nevertheless one or two of the figures found are of considerable value in themselves.

Every step in the migration of the cleavage cells to the periphery, their approach to the polar nucleus derivatives, the flowing together of the protoplasmic areas which surround each nucleus and the final apposition of the nuclei within the single protoplasmic area resulting, can be traced through closely seriated stages. Similarly, what appears to be a pair of polar nucleus derivatives may at times be seen in close proximity. However unless the chromosomes of the apposing·nuclei are close to full condensation, no definite conclusion can be reached as to the nature of the nuclei involved in either case. All of the figures show that the process takes place in either two or three cells, and a greater number has not been observed (Figs. 8 to 10).

It might be supposed that even during the resting phases the sizes of the fusing nuclei would suffice to identify them. And indeed it seems well established that when the two types of nuclei are at precisely the same stage, that of the polar nucleus derivative with its 15 chromosomes is slightly larger than a cleavage nucleus with 10. But it is practically impossible to exactly identify the phase of the nucleus during its preparatory phases. At the same time it has already been noticed that variations in the size of any one type of nucleus are extreme. The changes in size seem directly related to the condition of the contained chromatinic material and are such that the nuclear volume is smallest just after the formation of the nuclear wall at telophase, and largest immediately before the dissolution of the nuclear wall prior to the following division. Thus the increase in size of the female pronucleus between the telophase of the last maturation division and the time when the chromosomes are again almost fully condensed before the first segmentation division, are very considerable (Figs. 8a and 11, '23). Changes of size almost as great can be observed in the polar bodies prior to the formation of the polar nucleus and the polar nucleus derivatives. It is therefore manifestly impossible to arrive at any conclusion regarding the nature of fusing nuclei by simply comparing their size when it is considered that a further complication arises from the fact that apposing nuclei may be at entirely different phases (Fig. 7a and 10).

In spite of the very different phases that two or even three apposed nuclei may be in, it is apparent that a normal plate of chromosomes, which represents the summation of the numbers contained in each of the fusing nuclei, is finally attained. This may happen only when all of the nuclei are in a very definite generation of cells as has been pointed out in regard to the question of the number of divisions undergone by the polar nucleus. It is also a consequence of these observed facts regarding the varying phases of apposed nuclei, that the chromosomes of one or two of the nuclei will reach their full condensation prior to those of the other nuclei involved. Those first evolved must therefore be subjected to a suspension of further activity until those lagging behind have caught up. All of my figures make it

plain that condensation of chromosomes progresses regardless of the phase of an apposed nucleus, and that therefore the period of suspension of activities occurs when the chromosomes have been fully evolved.

Whether complete fusion of such nuclei is ever brought about before the condensation of chromosomes cannot be answered with certainty. A cytological demonstration would be next to impossible if the act is a very short one—say like the fusion of two soap bubbles to make a single larger one. That I have no stages showing such an act is therefore not complete proof that it does not occur. Nevertheless the normal course consists of a condensation of the chromosomes entirely independent of any other nucleus, and the fusion occurs only when the nuclear walls break down and permit an intermingling of all the chromosomes.

It is owing to the conditions brought out in the preceding paragraphs that a very good cytological demonstration of the act of fusing can be given. In Fig. 10 are shown three nuclei in apposition, and in the light of the numerical data they may safely be assumed to represent one polar nucleus derivative and two cleavage nuclei. Without the numerical data however, no such assumption would be justified. Fig. 11 on the other hand furnishes strikingly independent proof. Here there are 20 chromosomes almost fully condensed, and these show a slight trace of being arranged in two groups of 10 each. But in addition there are 15 chromosomes still in a more threadlike stage, and evidently at an earlier phase of condensation. The figure evidently represents a case in which the nuclei when coming into apposition were at different phases. The conclusion seems inescapable that here is represented the fusion of a polar nucleus derivative with two cleavage cells.

Spindles formed in the first division of these combination or fusion nuclei are apparently perfectly normal. Multipolar spindles are indeed encountered but little if any more frequently at this time than they are in the normal tissue of many animals. I am entirely at a loss to explain how the mitotic mechanism of two or even three combining nuclei is adjusted to the process of fusion. Certainly all the involved nuclei are capable of dividing perfectly independently. Bowen ('22) has recently pointed out a

similar case in *Loxa florida* wherè a fusion of cells is likewise un-accompanied by any irregularity in the mitotic spindle formation.

CONDITIONS IN OLDER EMBRYOS AND IN ADULTS.

As explained previously, the fusion process is limited chiefly to the period in which the blastoderm is laid down. When the latter is fully established, figures showing apposed nuclei in a single cytoplasmic area become very rare. Most of such figures arise from what is probably an accidental migration of yolk nuclei to·the periphery, for a few have been found in the blasto-derm as well as in the giant cell area. It is even doubtful whether in these isolated cases a fusion of nuclei is finally consummated, for no multiple cells have been found in the blastoderm cell region. The increase in the numbers of chromosomes in the giant cells, which is certainly still occurring at this time as well as later, is therefore due principally to a division of chromosomes unaccom-panied by a cytoplasmic division of the cell concerned (Fig. 18).

When after leaving the periphery the giant cells have become associated with the symbionts, mitotic figures are not so often found in them. Nevertheless they do occur, and successfully as far as the chromosomes are concerned. Cytoplasmic division which before the migration, was undoubtedly completed success-fully in some of the mitoses, is now completed less often. Only in this way can the relatively enormous numbers of chromosomes in the mycetocytes of the adult be explained. Further fusion is in these later stages practically eliminated, since most of the myceto-cytes are almost completely hedged in by the symbiont spheres which they harbor.

Buchner ('21) estimates the chromosome number in some of the mycetocytes as over 200. In this estimate I can only concur with him. There is in addition to this a decided increase in the individual size of the chromosomes, although this seems to be a variable feature in different mycetocytes (Fig. 19 and 20).

My material is not favorable for a detailed study of the spindle formation in these later mycetocytes. The centrosomes seem extremely small under even the most favorable circumstances, and special staining methods are difficult to apply to these as to other insect eggs. Apparently mitotic figures are normal now as

well as in embryonic stages and I am induced to regard Buchner's figure of a multipolar spindle as an exceptional case. That such may occur I have no reason for denying, and it is indeed strange that abnormalities are not the rule rather than the exception in all of the mycetocytes.

The size of the cells is not proportionate to the increased amount of chromatinic material. It is augmented considerably when compared with that of the giant cells in the time of first association with the symbionts, but never reaches the dimensions that one would expect from an examination of the contained chromosomes.

General Considerations.

Breest's and Strindberg's conclusions that mycetocytes arise from the yolk cells may safely be discarded as far as *Pseudococcus* is concerned. By Strindberg's own definition, yolk cells are cleavage cells which have been left behind after the general migration to the periphery to establish the blastoderm. The giant cells however, which are the direct progenitors of the mycetoctes, arise even before the migration of cleavage cells is complete, and therefore before the yolk cells have been established as such.

Pierantoni's conclusions have already been taken up in my previous paper ('23). Undoubtedly he is correct in his explanation that the cleavage cells wander in among the symbionts, but in *Pseudococcus* I consider this association, if such it can be called, as one of the most temporary nature. It is the natural consequence of the general peripheral migration of cleavage cells.

The whole series of developments, as here described, seems extraordinary. And yet, most of the stages regarded individually are not unprecedented; the processes involved have been described before in other forms.

A fusion of polar bodies and the persistence of the polar nucleus thus formed has been described in several polyembryonic Hymenoptera. The formation of a polar nucleus is therefore not the only case among insects. It is indeed only rarely that such instances are met, but the old assumption that polar bodies never develop in a normal case of embryology certainly does not hold. That the polar nucleus does not behave like the female pronu-

cleus is of course quite evident. In *Pseudococcus* for instance, its derivatives tend to stay at the periphery and do not sink into the egg as does the pronucleus. Nevertheless those inherent qualities in the latter which cause it to fuse with the male pronucleus, may be present to a certain extent also in the polar nucleus derivatives and cause them to combine with any cell that happens to come in contact with them. Certainly this tendency is not to be observed in the cleavage cells, for these are never found to fuse with each other under ordinary circumstances. It is found only in the polar nucleus derivatives, which can fuse both with each other and with the cleavage cells.

In much of their further behavior they are not anomalous at all. It is almost unnecessary to mention that in case of a great many animals and plants, complete fusion of the pronuclei may be delayed for some time. In such extreme cases as *Cyclops* (Rückert, '95; Haecker, '95) and *Cryptobranchus* (Smith, '19) the individuality of the two pronuclei may be traced even through the early cleavage stages. The failure of immediate fusion of two apposed nuclei in *Pseudococcus* is therefore not peculiar. As a matter of fact, it seems to be a rule in insects that the two pronuclei lie in apposition and the chromosomes of each are evolved independently of those of the other. It is only when nearly fully condensed that the nuclear walls break down and the chromosomes intermingle. Such seems to be the case in *Archimerus* (Morrill, '10), "goumi aphid" (Stevens, '06), *Trialeurodes* (Schrader, '20) and finally in the pronuclei of *Pseudococcus* itself.

Another aspect in the process of fusion of polar nucleus derivatives and cleavage cells is to be observed in the fact that two apposed nuclei may be in different phases. It has been explained that at such times the chromosomes of the nucleus in a more advanced phase are fully condensed but then enter on a period of suspended activity. During this period the chromosomes of the apposed nucleus or nuclei also become condensed and only then the common spindle is formed and the different sets of chromosomes are arranged in a single plate. The mechanism involved in this regulative process is not clear to me. But it may be stated that this aspect also is parallelled by the behavior of pronuclei

in several forms. Here may be mentioned *Lilium* (Weniger, '18), the "goumi aphid" (Stevens, '06), and once more the pronuclei of *Pseudococcus* itself. In the latter case I have mentioned ('23) the possibility that delay in the condensation of chromosomes in one of the two pronuclei may be connected with the peculiar chromosome conditions of the male. This is at best only a working hypothesis.

The present account makes it evident that, generally speaking, the fusion of the polar nucleus derivatives with migrating cleavage cells is a more frequent occurrence than I had previously supposed. It therefore seems best to apply the name "polar nucleus derivative" only to the cells carrying 15 chromosomes, which are products of the division of the original, single polar nucleus. In my previous paper ('23) this term was applied somewhat indiscriminately to cells arising from division of the polar nucleus as well to some of those that had already undergone fusion with other cells and therefore contained a multiple number of chromosomes. The latter type of cell has been called "giant cell" throughout the present paper and of course includes cells arising from the fusion of polar nucleus derivatives among themselves, as well as with cleavage cells. The distinction between the giant cells and the polar nucleus derivatives is thus made a very definite one. Giant cells that have entered into association with the symbionts therewith become mycetocytes.

No attempt has been made here to discuss the exact relations between the insect body and the symbionts harbored by it. It should be pointed out however that the mycetocytes are insect cells. But they are in a measure extraneous to the organization of the body of the insect and even their actual connections with the latter are confined to branches of the trachea. Even during development they do not stand in a more direct relation to the various organs of the embryo than do the symbionts themselves. Their physiological importance nevertheless may be considerable; but this is a problem in itself.

I realize that the difficulties of the case are not removed by listing parallel instances of various stages—as I have done in this discussion. Such a proceeding however does serve to emphasize that many of the questions brought up by the investigation are

identical with problems that have troubled the cytologist for years.

SUMMARY.

1. The first and second polar bodies of *Pseudococcus* undergo fusion and form a polar nucleus. This contains 15 chromosomes.

2. The polar nucleus divides several times (probably a definite number of times) giving rise to the polar derivatives.

3. The polar derivatives may fuse with either migrating cleavage cells or with each other to form the giant cells. The numerical data furnished by chromosome counts as well as the purely cytological evidence support each other in arriving at this conclusion.

4. The giant cells migrate from the periphery to the symbionts to enter into association with these. When this process has been completed, the giant cells are known as mycetocytes.

5. Discussion regarding the nomenclature of the cells involved in these phenomena. Statement of the problems presented during the various stages of the investigation.

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EXPLANATION OF PLATES

All figures have been drawn at table level, using a camera-lucida. Tube length = 160 mm., 2 mm. Zeiss oil immersion objective, and No. 12 X compensating ocular. No reduction has been made in the reproductions.

PLATE I. (all figures of *Pseudococcus citri*).

FIG. 1. Chromosomes of the fertilization nucleus.

FIG. 2. Blastoderm cell during early stage in the blastoderm formation.

FIG. 3. Blastoderm cell at time when blastoderm has become complete and giant cells have begun to migrate to the symbionts.

FIG. 4. The polar nucleus.

FIG. 5. One of two daughter cells derived from the first division of the polar nucleus, *i.e.*, one of first polar nucleus derivatives.

FIG. 6. One of two daughter cells derived from the division of one of the first · polar nucleus derivatives. Four derivatives present in the egg at this time.

FIG. 7. Telophase of the third division of the polar nucleus. Eight derivatives in the egg when this division is complete.

FIG. 7a. One of four polar nucleus derivatives with fifteen chromosomes almost condensed for the next division. Cleavage cell in resting stage coming into apposition.
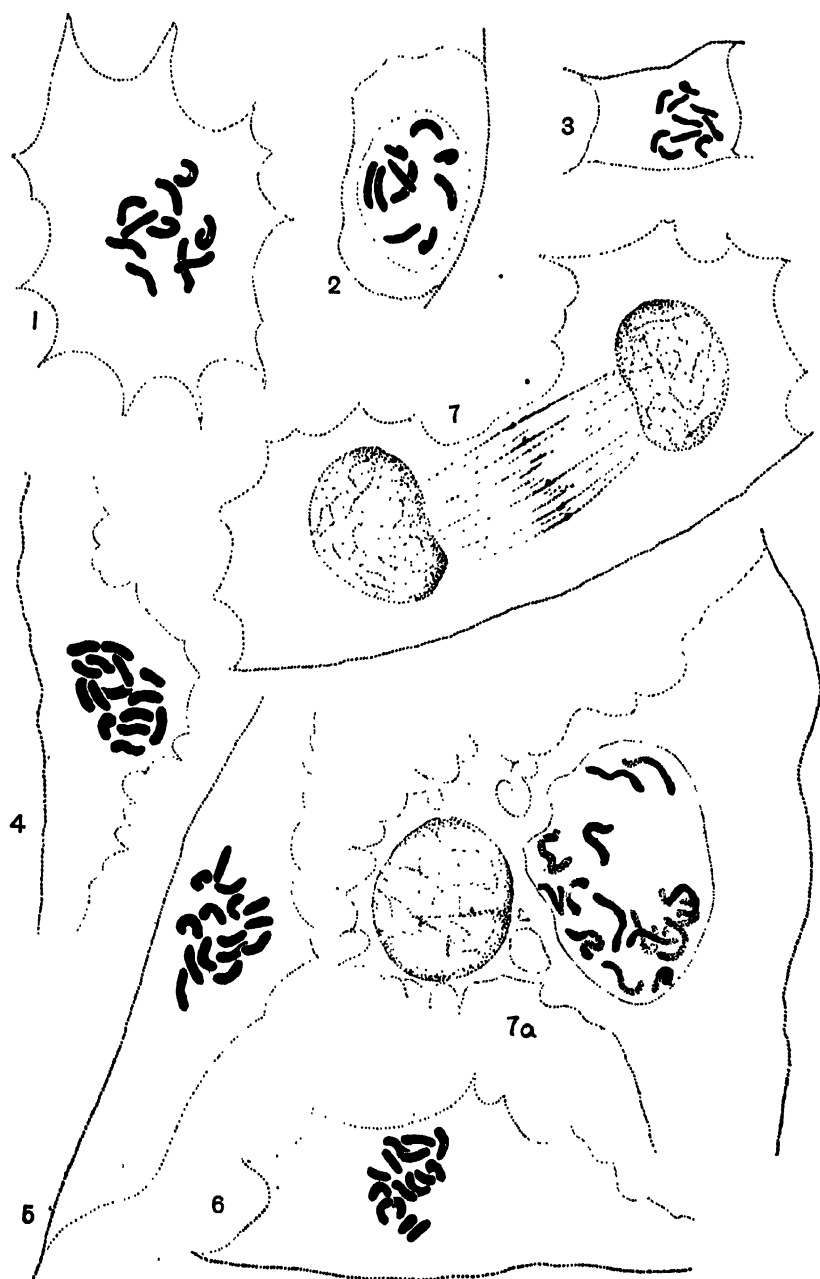
PLATE II.

FIG. 8. Cleavage nucleus approaching a polar nucleus derivative. Cytoplasmic area fusing. (*P. maritimus.*)

FIG. 9. Two nuclei in apposition. Not certain whether both are polar nucleus derivatives. (*P. citri.*)

FIG. 10. Three nuclei in apposition. The two smaller with chromatin slightly more condensed than that of the larger nucleus. Probably two cleavage nuclei and a polar nucleus derivative. (*P. maritimus.*)

FIG. 11. Nucleus showing 20 chromosomes almost fully condensed and 15 at a slightly earlier phase. The 20 condensed chromsomes seem to be arranged in two groups of 10 chromosomes each. Probably originated from two cleavage nuclei and a polar nucleus derivative. (*P. maritimus.*)

THE ENDOCRINE SYSTEM OF *TYPHLOMOLGE RATHBUNI*.

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The endocrine system of *Typhlomolge rathbuni*, the blind Texan cave salamander, has been a matter of controversy for some time. In order to clarify some of the points under discussion, I have made serial sections of the region of the lower jaw, throat, and heart of 5 specimens, and the entire head of 2 specimens of this animal. The specimens were captured in Texas, as described in a previous article.[1] Six of them died during the trip from Texas to New York, one after 14 months of captivity in the laboratory. Since they are preserved after death, only the anatomical features of the various organs can be studied. A histological study must be postponed until suitable material can be secured.

THE THYROID.

Emerson[2] was the first one to call attention to the possible absence of the thyroid gland in *Typhlomolge*. In 1905 she examined sections through the head of one specimen and was unable to find a thyroid. At the time Emerson published her paper the interest in the endocrine system of amphibians was very slight and her paper remained unknown to most biologists. In several of my papers on the thyroid function of salamanders I have called attention to Miss Emerson's interesting findings, which I had recognized to be correct. Soon after my return from Texas in 1916, I sectioned one of the *Typhlomolge* captured there and found the thyroid absent.

But at the 1921 Christmas meeting of the Anatomists, Swingle, apparently unacquainted with the literature on these facts spoke of the thyroid of *Typhlomolge* as a matter of fact and claimed to have isolated and observed this organ under the microscope.

[1] Uhlenhuth, E., *Biol. Bull.*, 1921. XL., 73.
[2] Emerson, E. T., *Proc. Soc. Nat. History, Boston*, 1905, XXXII., 43.

Although I mentioned my own findings, Swingle's very definite claims made the correctness of my observations doubtful, and even Doctor Wilder, from whose laboratory Emerson's paper was published, was ready to admit the possibility of an oversight on the part of Miss Emerson.

Immediately after my return from this meeting, I made sections of the 7 specimens discussed in this paper, and upon examination of the first one I was convinced that I was correct. In response to a letter in which Swingle admitted that the organ which he had claimed to be a thyroid was another vesicular organ, I communicated my new observations to Mr. Swingle. Neither this communication nor the incident at the Anatomists' meeting has been mentioned in an account recently published by Mr. Swingle in which he[1] states that 3 specimens examined by him possessed no thyroid.

The examination of the 7 specimens, together with previous findings, shows that while some specimens of *Typhlomolge* are without even vestiges of the thyroid, others possess epithelial structures, evidently undifferentiated thyroid rudiments whose development was inhibited by an unknown factor.

Before describing these rudiments, the location of a normal thyroid may be briefly referred to. For comparison I shall use the thyroid of the Ambystomidæ, which may be called representative of a normal salamander thyroid. In the Ambystomidæ, the median thryroid rudiment splits up into two epithelial cell masses, which migrate in a posterior, lateral and largely ventral direction until, in *Ambystoma opacum*, they are closely attached to a large lymphatic space (Fig. 1). This space is located in the interstitial space formed by the muscles which surround the first gill arch, ventral and median to the epibranchial of the first gill arch. In other species the thyroids may be located slightly further posterior, but always the lymphatic space where it is in touch with the thyroid is adjacent also to the anterior cardinal veins. At the site where the ventral end of the thyroid is attached to the lymph space, the anterior cardinal vein comes into close contact with this space, leaving there the thyroid, at its ventral and posterior end, as a large vessel into which collects the blood from the interfollicular rete of the thyroid.

[1] Swingle, W. W., *Jour. Exp. Zoöl.*, 1922, XXXVI., 397.

In addition to the main portions of the thyroid, most specimens possess accessory thyroids.   These develop from small cell groups which during migration become detached from the main portions and thus mark the path along which the main portions
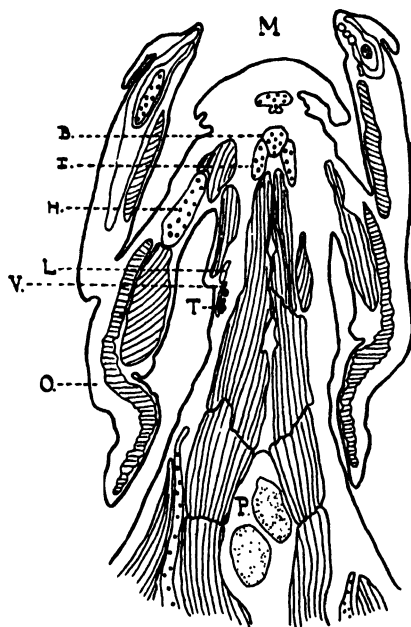
In reproduction figures 1 to 8 have been reduced by one third, figures 9 and 10 by slightly more than one third.

FIG. 1.   Location of main portion of right thyroid in an advanced larva (59.2 mm. total length) of *Ambystoma opacum; I,* first gill arch; *B,* first basibranchial; *H,* hyoid; *L,* lymph space; *M,* cavity of mouth; *O,* operculum; *P,* pericard; *T,* thyroid; *V,* anterior cardinal vein.

migrate. Their location varies greatly. They are located usually anterior and may be either ventral or dorsal or, in case of several accessories, both ventral and dorsal to the main portions. Or they may be at one level with the main portions. They consist either of one or several median rudiments located in the median interstitium of the muscles ventral to the basibranchials of the visceral skeleton (genio-hyoideus) or of two lateral portions, one on each side, which either are attached to the sides of the basi-branchials or are located in the interstitia of the muscles lateral and ventral to the basibranchials. In some Ambystomidæ

(*A. tigrinum*) the lateral portions may develop into normal thyroids of considerable size.

The thyroid rudiments of *Typhlomolge* occupy a position closely resembling the location of the various thyroid portions of the Ambystomidæ.

Typhlomolge 1, a sex-mature animal of 111 mm. total length and 58.2 mm. body length, does not possess even vestiges of a thyroid. The region of the lower jaw, throat, and heart was sectioned into a complete series; no section is missing. The anterior cardinal vein was followed in its entire course, the visceral cartilages and muscles were carefully searched through, but no traces of a thyroid could be found.

Typhlomolge 3, a small, apparently young, animal of 57.7 mm. total length and 32.6 mm. body length possesses a median thyroid rudiment. It is entirely detached from the pharyngeal epithelium and partly imbedded into the muscles just ventral to the basibranchial (genio-hyoideus). It is located between the attachments, to the basibranchial, of the hyoids and first gill arches and just anterior to the latter ones. The tissues are not well preserved, but the rudiment is seen to consist of several vesicles possessing epithelial walls and containing no colloid. No other epithelial structures were found, although the anterior cardinal vein was searched in its entire course down to the *Ductus cuvieri*, and the lymphatic space, the muscles, and cartilages of the visceral skeleton were carefully inspected.

In *Typhlomolge* 7, an animal of 75. 6 mm. total length and 43.1 mm. body length, a median rudiment is attached to the ventral surface of the muscles just ventral to the basibranchial, about in the middle between the attachments of the hyoid and first gill arches (Fig. 2). It consists of a single solid cell mass of epithelial structure (Fig. 3). In the center a network-like structure is noticed, produced very likely by the cell walls of the clear inner cell ends; the same structure is frequently found in tangential sections through the walls of the follicles of normal thyroids. No colloid is contained in this rudiment. In addition to the median rudiment, two lateral rudiments are present, one on each side. The anterior ends of these rudiments are located near the connection between ceratobranchial and epibranchial of the first

gill arch and median to this arch (Fig. 4). They extend in a posterior direction; the posterior end approaches closely the wall of the lymph space, but does not come in contact with it. It is
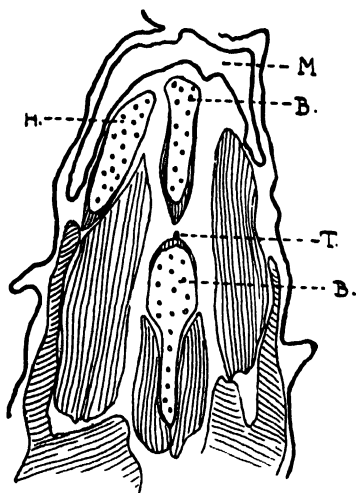

FIG. 2. Location of median thyroid rudiment in *Typhlomolge* 7. *B*, basibranchial; *H*, hyoid; *M*, cavity of mouth; *T*, thyroid rudiment.
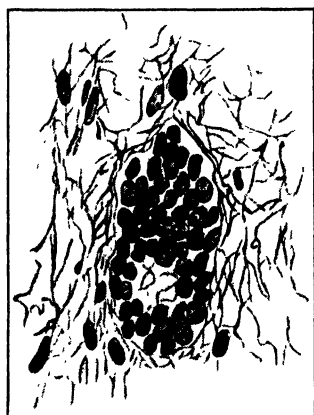

FIG. 3. Median thyroid rudiment of *Typhlomolge* 7. × 340.

evident from this account that the lateral rudiments of *Typhlomolge* occupy a location similar to that of the main portions of the thyroid in *Ambystoma opacum*. They are, however, located further anterior, as if they had stopped migrating before attaining the definite position. Moreover, the place where the anterior cardinal vein passes the lymph space is located considerably more ventral; therefore, the lateral rudiments are nowhere in contact with this vein. It is indeed impossible to see any vessels supplying the thyroid rudiment;
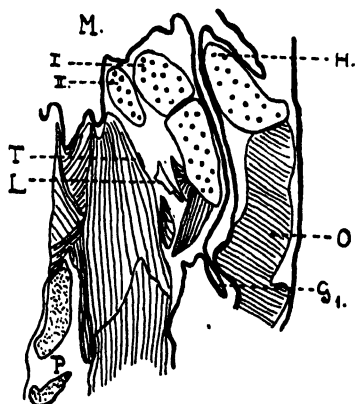

FIG. 4. Location of lateral thyroid rudiment of *Typhlomolge* 7. I., first gill arch; II., second gill arch; *G*₁, plate of first gill; *H*, hyoid; *L*, lymph space; *M*, cavity of mouth; *O*, operculum; *T*, thyroid rudiment.

if there are any they must be very small. The lateral rudiments consist of a series of tiny epithelial cell masses; some of them are solid, others are hollow, but none of them contain colloid.

In *Typhlomolge* 6, the smallest and probably youngest animal (56.0 mm. total length and 32.0 mm. body length), no median but one lateral rudiment on each side is present. They consist of a series of cell masses (Fig. 5), some of which are solid while others contain a lumen. Colloid is absent in all of them. The location is similar to that of the lateral rudiments of the previous specimen. In particular they do not touch the lymph space and are situated dorsal to the place where the anterior cardinal vein comes into contact with this space. They are without a blood supply resembling that of a thyroid.
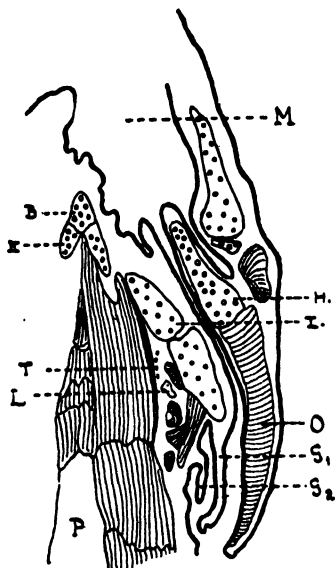
In *Typhlomolge* 2, an animal of 77.5 mm. total length and 43.6 mm. body length, only the lateral rudiments are present. Their location is the same as that of the lateral rudiments described above. Instead of being broken up into several separate cell masses, each of them has the shape of one continuous epithelial cell tube possessing a narrow lumen (Fig. 6).

FIG. 5. Location of lateral thyroid rudiment of *Typhlomolge* 6. I., first gill arch; II., second gill arch; *B*, basibranchial; *G*₁, blade of first gill; *G*₂, blade of second gill; *H*, hyoid; *L*, lymph space; *M*, cavity of mouth; *O*, operculum; *P*, pericardium; *T*, thyroid.

In *Typhlomolge* 5, an animal of 66.0 mm. total length and 36.5 mm. body length, only one lateral rudiment, the left one, is present. It is located near the connection between the ceratobranchial and epibranchial of the first gill arch, median to it and anterior to the location of a normal thyroid of *Ambystoma opacum*. It is composed of a small number of vesicles (Fig. 7) which, instead of being arranged in an antero-posterior row as in the other specimens, are crowded together in one place. The

vesicles have a distinct epithelial lining and are hollow; they do not contain colloid.   In addition to this rudiment, *Typhlomolge* 5 possesses another one of sim-
ilar structure, located on the same side but more median.   It is attached to the left side of the muscle just ventral to the basi-branchial and just anterior to where the first gill arch connects with the basibranchial.   Thus it has very nearly the same location as the median rudiment of other animals, but is displaced slightly to the left side.   Apparently the primary median rudiment of this animal split into two rudiments; one of them, the left one, moved into its normal   position.   The right one not only failed to do so, but was dragged along a short distance by the left rudiment before complete separation was

FIG. 6.   Left lateral thyroid rudiment of *Typhlomolge* 2.   X 340.

FIG. 7.   Left thyroid rudiment of *Typhlomolge* 5.   X 340.

accomplished, and thus was dislocated from its primary median position to wards the left side.

The 6 specimens described so far died on the way from Texas to New York, shortly after they had been removed from the caves. The seventh animal, *Typhlomolge* I, a specimen of 97.5 mm., died after it had been kept alive in the laboratory for 14 months. The largest part of this time (12 months) it lived at a temperature of 15° C. and in darkness; for the last two months it was kept in an aquarium stocked with plants, small crustaceans and young tadpoles, in daylight and at a temperature of approximately 22 to 25° C. The sections of this specimen are greatly torn and I am not sure that our inability to find a median and left lateral rudiment is due to the absence of these organs and not to the poor condition of the sections. On the right side, however, a lateral rudiment is present. It is located near the lymph space, anterior and dorsal to the location of the main portion of the thyroid of *Ambystoma opacum* and resembles more closely a thyroid structure than the rudiments of the specimens described previously. It consists of a small number of hollow vesicles which compose an elongate, egg-shaped organ possessing a connective tissue capsule and hence impressing one as a distinct and individual organ. The walls of the vesicles are of epithelial character; but no colloid is contained in the lumen of the vesicles (Fig. 8).

Summary: Although *Typhlomolge*, in its advanced stages, does not possess an organ resembling the normal thyroid of a salamander, epithelial structures are found which indicate that in the young embryo of this animal the thyroid rudiment forms in a similar manner as in other amphibians. This rudiment, however, for some reason, fails to develop into a thyroid. In some animals, development ceases after the median epithelial outgrowth has separated from the pharyngeal epithelium and the rudiment remains a single vesicle. In other cases it may partly split up into lateral portions which, as in other salamanders, move in a posterior direction and, in some instances, may approach closely the lymph space; but they never reach the anterior cardinal vein. The unsplit part of the median rudiment may retain its median position and primitive vesicular structure; the lateral portions

may either develop into a continuous epithelial tube or may break up into a series of solid or hollow cell masses.   In some animals no median rudiment is found; if this condition develops in consequence of a complete splitting-up of the median rudiment or of later degeneration of the median rudiment, can be decided only by studying the embryology of this animal.   In one specimen no vestiges of the thyroid are present at all.   If Emerson's and Swingle's statements are not due to an oversight, on their part, of the inconspicuous epithelial structures, there are now 6 specimens of *Typhlomolge* known which did not possess any vestiges of the thyroid.   One was described by Emerson, 3 by Swingle, one was found previously by me and the sixth animal is the one described in this paper.   Either no thyroid rudiments developed in these six animals, or they were reabsorbed shortly after they had developed.

It is certain that the thyroid rudiments of *Typhlomolge* which do persist retain permanently a primitive epithelial structure and fail to develop, among many other structures of a normal thyroid, a venous rete and the colloid.
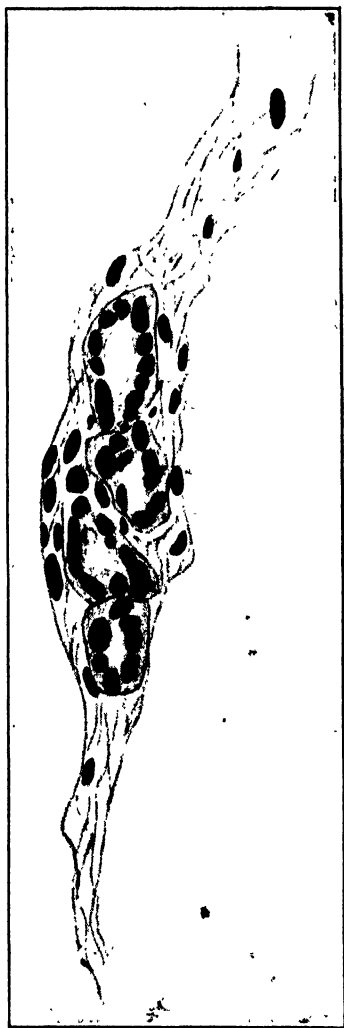

Fig. 8.   Right thyroid rudiment of *Typhlomolge* I.   X. 340.

OTHER ENDOCRINE ORGANS.

It may be briefly mentioned that the thymus glands, the hypophysis, and the postbranchial body were found to be present in

every specimen. Like other salamanders, *Typhlomolge* possesses
3 pairs of thymus glands; in one animal they were found fused
into two large glands, one on each side. This condition is fre-
quently met with in adult salamanders.

The postbranchial body, although, on a whole, it resembles
this organ in other salamanders, shows certain peculiarities
(see Baldwin's paper [1] for a description of this organ). Its struc-
ture is very similar to that found in *A. opacum;* in particular, it
is found only on the left side. It is an epithelial structure of the
shape of a tube possessing, in places, epithelial diverticula. A
lumen is frequently absent, while in *A. opacum* and other Amby-
stomidæ this organ possesses often a very condiserable lumen.
The cephalic end of the organ is located in the pharyngeal epithe-
lium with which it connects near the place where in Amby-
stomidæ the *Aditus laryngeus* is situated. In the Ambystomidæ
the posterior end of the organ is often very large as compared to
the thin duct-like anterior end and is located on the left side of
the pericardium, posterior to the fourth aortic arch. Frequently
it is closely attached to the pericardium and posterior wall of the
fourth aortic arch. In *Typhlomolge* the fourth aortic arch is
missing; the postbranchial body attaches itself to the third aortic
arch. In some animals it reaches back to the heart and is found
attached to the pericardium. Its posterior end, however, does
not attain the size which this part is found to attain in *Ambystoma*.
Moreover, in some specimens the organ remains short, extending
backward only to the middle between pharynx and pericardium.
In these cases its posterior end becomes attached to the wall of
the third gill arch approximately half-way between the pericard-
ium and the entrance of the arch into the gill blade of the third
gill. It seems that the postbranchial body of *Typhlomolge*,
although it possesses, on the whole, the structure of the normal
organ of the Ambystomidæ, shows sometimes signs of develop-
mental inhibition.

The hypophysis was studied only in two animals and only in
transverse sections. Like the hypophysis of the Ambystomidæ [2]
it is composed of 4 parts, the pars anterior proper, the partes
tuberales, the pars intermedia and the pars nervosa. In the pars

[1] Baldwin, F. M., *Jour. Morph.*, 1918,XXX., 605.
[2] Atwell, W. J., *Anat. Record*, 1921, XXII., 373.

anterior, the largest part of the entire organ, the individual tubes are discernible more distinctly than in the Ambystomidæ. They take an antero-posterior course and are arranged parallel to each other (Fig. 9). In the spaces separating the individual tubes,

FIG. 9. Transverse section through the hypophysis of *Typhlomolge* 7. *b*, blood vessel; Ɔ-infundibulum; *i.w.*, infundibular wall; *L*, lumen of individual tube: *p.t.*, left pars tuberalis (of the right pars tuberalis this section contains only the connective tissue sheet of the ventral surface). *Sh*, connective tissue sheet. *t*, individual tube of pars anterior. × 340.

large blood vessels are located. Frequently the individual tubes possess a distinct lumen; the nuclei of the cells are located at the distal end of the cell, towards the lumen of the tube. The cell walls are sometimes very distinct. The anterior end of the pars anterior continues into two lateral processes, the partes tuberales,

which, like in other salamanders [5, 6, 7,] are continuous with the pars anterior and attach themselves to the ventral wall of the infundibulum (Fig. 9). The partes tuberales of *Typhlomolge* are apparently smaller than in the adult *A. opacum* and resemble in size the partes tuberales of a larvæ of *Ambystoma opacum* of about 55 mm. total length and showing no signs of metamorphosis as yet. The pars intermedia of the amphibians cannot well be discriminated from the anterior part in transverse sections. But from such sections as shown in Fig. 10, it would seem that the dorsal part of the pars intermedia is bilobed, the two lobes being separated by a median antero-posterior space. The pars nervosa, as in other salamanders, consists in a thickening of the wall of the infundibulum where the pars intermedia is attached to it (Fig. 10). In comparing the pars nervosa of *Typhlomolge* with that of other salamanders, Haller's description [7] of the pars nervosa of *Proteus anguineus* is of interest. According to this author, the pars nervosa of Proteus is hardly differentiated from the rest of the infundibular wall. In *Typhlomolge* the pars nervosa is not only well-differentiated, but seems to be larger and more sacculated than is the case in *Ambystoma opacum* (Fig. 10). Summarizing
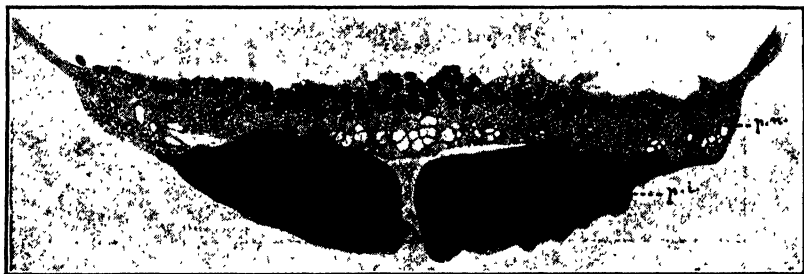

FIG. 10. Transverse section through dorsal regions of hypophysis of *Typhlomolge* 7. *p.i.*, pars intermedia; *p.n.*, pars nervosa. × 340.

the description of the hypophysis of *Typhlomolge*, one may say that it resembles closely the hypophysis of other salamanders. In particular, it does not seem that the hypophysis of this species presents indications of an atrophic state, although, with a larger amount of material at our disposal we might find that the partes tuberales of *Typhlomolge* are of the size of a larval organ.

[6] Haller, B., *Morph. Jahrb.*, 1898, XXV., 30.

[7] Haller, B., *Arch. Mikr. Anat. und Entwicklgsg.*, 1909, LXXIV., 812.

DISCUSSION.

The factor which led to the inhibition of the thyroid development is unknown. The researches of Leo Adler [8] and Bennett M. Allen [9] showed that extirpation of the hypophysis in amphibians inhibits the development of the thyroid. One could imagine that defective development of the hypophysis might have been the immediate cause of the inhibition of the thyroid development in *Typhlomolge*, but so far, no abnormalities in the structure or mutual relations of the various parts of the hypophysis have been discovered, which could account for the thyroid atrophy of *Typhlomolge*—a phenomenon singular in the vertebrates.

It is not known whether the atrophy of the thyroid of *Typhlomolge* is only one of the results caused by certain factors which lead to the inhibition of the general development of this animal, or whether the inhibition of the thyroid was primary to other developmental inhibitions. As pointed out in previous papers [10] the athyroidism of this species possesses special interest, because in ·the same species metamorphosis also is suppressed. I have assumed, in a purely tentative manner and in order to obtain a basis for further experiments, that the latter phenomenon is the direct result of the lack of the thyroid. In the absence of adequate experiments and in the light of the well known fact that extirpation of the thyroid inhibits the amphibian metamorphosis [11], [12], this explanation still seems to be the most feasible one.

Swingle,[13] in a recently published article, takes occasion to criticise my attitude, outlined above, towards the problem of neoteny in *Typhlomolge*. He has made certain observations confirming the existence of a releasing mechanism in salamanders. Regarding the facts communicated in this article, these surely should be welcome to the writer of the present article, in so far as they led Swingle to exactly the same conclusion as that at which I arrived as early as 1919. But certain statements made in this paper are apt to give rise to misunderstandings. To prevent these a discussion of Swingle's paper seems desirable.

[8] Adler, L., *Arch. Entwcklgsmech. d. Org.*, 1914, XXXIX., 21.

[9] Allen, B. M., *Biol. Bull.*, 1917, XXXII., 117.

[10] Uhlenhuth, E., *Endocrinology*, 1922, VI., 102.

[11] Allen, B. M., *Science*, 1916, N. S. XLIV., 755.

[12] Hoskins, E. R., and Hoskins, M. M., *Jour. Exp. Zoöl.*, 1919,XXIX., 1.

[13] Swingle, W. W., *Jour. Exp. Zoöl.*, 1922, XXXVI., 397.

Throughout his paper Swingle attempts to create in the reader's mind the impression that in my previous work I have laid too much one-sided emphasis on the thyroid gland as the only organ potent in the amphibian metamorphosis. This attitude is perplexing in view of the circumstance that I have been able to disclose facts demonstrating the existence of a releasing mechanism outside of the thyroid and in view of the other circumstance that Swingle received the discovery of this "releasing mechanism" when communicated by the writer in 1919 [14] in the following way [15] (p. 600): "Uhlenhuth, while accepting the conclusions stated regarding the relation of iodine to amphibian metamorphosis, thinks that still another substance is needed to cause the thyroid gland to excrete the iodine necessary for metamorphosis. This hypothetical factor he terms excretor substance and thinks that it is evolved during the growth processes of the organism. The assumption of an excretor substance obscures rather than clarifies the already sufficiently complicated problem of amphibian metamorphosis."

As to Swingle's criticism regarding the omission, on my part, of the possible defectiveness of the releasing mechanism in the explanation of the neoteny of *Typhlomolge*, it should be pointed out that the writer of this article has, reluctantly, refrained from suggesting this possibility, because no experiments suggesting it have been—or are today—available. As to the actual interpretation of the neoteny of *Typhlomolge* and as to my attitude [16] towards Jensen's [17] experiments which showed that adult *Proteus* and *Necturus* do not metamorphose upon thyroid administration, the following statements may be made: (1) the interpretation of this phenomenon as given in my previous papers [16] does not form an integral part of the theory of a releasing mechanism; (2) the most pertinent problem in regard to the neoteny of *Typhlomolge* was the question whether or not this animal possesses a thyroid gland proper, a question to the solution of which Swingle has contributed nothing, as will become evident from a perusal

[14] Uhlenhuth, E., *Jour. Gen. Phys.*, 1919, I., 473.

[15] Swingle, W. W., *Jour. Gen. Phys.*, 1919, I., 593.

[16] Uhlenhuth, E., *American Naturalist*, 1921, LV., 193.

[17] Jensen, C. O. *Oversigt kgl. Danske Vidensk. Forhandl.*, Copenhagen, 1916, No. 3, 251.

of the introduction to this article and of the facts described above; (3) the lack of an effective releasing mechanism may have been the primary cause of the neoteny of *Typhlomolge*, but so far nothing as to this effect can be quoted; (4) the question of whether or not *Typhlomolge* and phsyiologically similar species, such as *Proteus* and *Necturus*, possess, in their present state, the ability to metamorphose upon thyroid administration is a problem entirely aside from the rôle of the releasing mechanism and has been considered so in the writer's previous papers. (It is possible that permanent suppression of the thyroid function over long periods may cause complete loss of the reactivity of the organism. The demonstration of such complete loss, however, does not decide the question as to whether primarily the thyroid ceased to function, in *Typhlomolge*, on account of a defective releasing mechanism); (5) it has not been proved as yet that *Typhlomolge*, *Proteus*, and *Necturus* have completely lost their ability to react to the thyroid hormone. It was merely this point which gave occasion for the criticism of Jensen's work. Jensen [17] exposed only the adult specimens of *Proteus* and *Necturus* to the action of the thyroid hormone. That he did not succeed in enforcing metamorphosis does not necessarily mean that the responsiveness of these animals has been completely lost. In order to show that *Necturus*, *Proteus*, and *Typhlomolge* could not metamorphose even if they were in the possession of a complete and normal thyroid mechanism, the young larvæ or even the parents at the time of development or ripening of the ova and spermatozoa may have to be subjected to thyroid administration. Swingle has merely repeated Jensen's experiments on *Necturus*, without modifying Jensen's technique. Like Jensen, he did not use the young larvæ, but the adult animals. Nowhere in Swingle's paper, however, can there be found any reference to Jensen's experiments on *Necturus*.

The same attitude is met with in Swingle's paper regarding the releasing mechanism. Although no progress beyond the present state of the problem has been accomplished, there is, in Swingle's article, no mention made anywhere of previous work on the same problem.

That the thyroid mechanism of salamanders consists of two physiologically distinct parts was found by the writer of this

paper as early as 1919.[14] The discovery of the factor necessary to release the thryoid hormone was summed up in the following statement,[14] (p. 476): "Hence, besides iodine, still another substance is needed in the amphibian metamorphosis; namely the 'excretor substance' which causes the thyroid to excrete the stored-up iodine." Swingle's statement [15] (p. 600), made in 1919 in reference to this work and quoted above, shows that he did not recognize the existence of such a releasing factor.

Since organs of internal secretion or any other organs would manifest themselves physiologically in a manner essentially similar to a substance and since it seemed undesirable to reflect in the term applied to the releasing factor upon any preconceived theory, the term "excretor substance" was replaced later on by the term "releasing factor" [16] (p. 207) and "releasing mechanism" [10] (p. 112), both of them implying merely the function by which this factor manifests itself and which was actually observed.

Since the first communication was made my experiments were continued and it was shown, for 3 different species of salamanders, that in low temperature metamorphosis is greatly retarded in proportion to general growth, while the development of the thyroid gland shows no such retardation. It was concluded from this fact that since the thyroid developed at a normal rate in proportion to general growth the development of the releasing mechanism was retarded. Several papers were published in regard to this problem and the difference between the retardation of the thyroid and the releasing mechanism in response to the same degree of lowered temperature was explained by assuming a lower temperature coefficient for the thyroid than for the releasing mechanism. In 1921 the results of this work were summarized in the following statement [16] (p. 206): "The most conspicuous character in the salamander metamorphosis is the fact that although it certainly is dependent on the thyroid hormone, it does not necessarily take place in larvæ whose thyroid is mature. This can only mean that two factors are required in order to bring about the metamorphosis of salamander larvæ, namely a mature gland and a factor which releases the thyroid hormone from the follicles of the gland."

Further confirmation of the existence of a releasing mechanism

has been found in the iodine experiments.[10, 18, 19, 20]   Administration of an excess of inorganic iodine does not enforce the metamorphosis of salamander larvæ,[10, 20] yet the elaboration of the colloid is accelerated by iodine feeding. This result was to be expected if the release of the hormone does not depend on the quantity of hormone developed in the follicles of the thyroid but is controlled by a particular releasing mechanism.

The results outlined above were checked also by histological sections of large numbers of thyroids of normal and experimental animals. Although the publication in full of this work has been postponed in order to assure greater completeness, single results have been referred to in various papers and have been demonstrated to colleagues and before meetings. In every case it was found that the elaboraion of the colloid and the excretion of it are two distinct and independent processes, physiologically as well as structurally. Elaboration of normal colloid is frequently met with in cases of inhibition of metamorphosis and in normal larvæ long before metamorphosis, and, in this case, is combined with complete absence of the structures characteristic of the excreting stage of the thyroid. This relation has been interpreted as further testimony in favor of the existence of a releasing mechanism.

I must also refer here to Swingle's criticism of my iodine experiments, since, if correct, it would question the value of these experiments as supporting the theory of the releasing mechanism. My experiments [10, 20] showed that, contrary to anuran larvæ, in the larvæ of salamanders metamorphosis cannot be enforced by the administration of inorganic iodine. The bearing of this fact upon a general theory of the rôle of iodine in the specific effect of the thyroid hormone has been outlined in detail in two previous papers.[10, 20]   Swingle's general attitude in his paper tends to create the impression (1) that I have claimed "iodine has nothing to do with the axolotl metamorphosis"[13] (p. 417) and (2) that somewhere in my papers are to be found statements to the effect that organic iodine compounds cannot enforce the metamorphosis of the axolotl and other salamanders.

[18] Uhlenhuth, E., *Jour. Gen. Phys.*, 1922, IV., 319.

[19] Uhlenhuth, E., BIOL. BULL., 1921, XLI., 307.

[20] Uhlenhuth, E., BIOL. BULL., 1922, XLII., 143.

As to the first point I should like to refer the reader to the following statement,[10] (p. 114) into which my results on the rôle of iodine were summarized: "That iodine if supplied in excess does not produce metamorphosis of salamander larvæ does not mean, according to what has been said above, that it is not necessary in the metamorphosis of salamanders. Very likely if larvæ of salamanders would be raised on an iodine-free diet and kept in iodine-free water, metamorphosis could not take place."

Regarding the second point Swingle quotes against me his own experiments [13, 21] in which he thinks he has shown that 3–5 di-iodo-tyrosine can enforce metamorphosis of thyroidectomized axolotls, and Jensen's experiments (22) with iodized proteins. Neither Swingle's own experiments nor Jensen's experiments referred to have proved that inorganic iodine can be utilized directly by the axolotl tissues to elaborate the thyroid hormone. The facts regarding the influence of inorganic iodine on the axolotl metamorphosis are, however, widely different from what Swingle would like them to be.

In the first place, Jensen has not only not shown that inorganic iodine does enforce metamorphosis of the axolotl, but on the contrary has shown that inorganic iodine as such is ineffective in the axolotl metamorphosis. In one of his papers, Jensen [23] points out that the effectiveness of thyroid preparations in enforcing the axolotl metamorphosis does not correspond to the iodine-content of these preparations. In a personal conversation, Professor C. O. Jensen told me that he had tested the action of inorganic iodine, but found it ineffective in enforcing the àxolotl metamorphosis. Jensen's experiments are therefore entirely in accord with my own experiments. Moreover, Professor Jensen's experiences which are well in accord with my own observations may serve as a warning against the reliability of those experiments which resulted in "enforced metamorphosis" of the axolotl. Among Professor Jensen's strains of the European race of the axolotl there were, in the beginning, animals which gave rise to offspring 50 per cent. of which would metamorphose

[21] Swingle, W. W., *Science*, 1922, N. S., LVI., 720.

[22] Jensen, C. O., *Compt. rend. Soc. Biol.*, 1920, LXXXIII., 315; 1921, LXXXIV. 423; 1921, LXXXV., 391.

[23] Jensen, C. O., *Hospitalstidende*, 1920, LXIII., 505.

spontaneously. Early in his work he began to select carefully individuals which produced 100 per cent. neotenous larvæ.

Swingle also quotes the experiments of Huxley and Hogben,[24] and of Hirschler [25] against me. What Huxley and Hogben really found, however, is that inorganic iodine does not enforce the metamorphosis of axolotls. There are still Hirschler's experiments; these are represented by "one" successful experiment. The total number of Hirschler's experiments on inorganic iodine in relation to axolotl metamorphosis is "two." One animal was given an intraperitoneal injection of iodoform; it died before a conclusive result was obtained. The other animal received an injection of iodine dissolved in potassium iodid; it metamorphosed completely. But the animal illustrated, as a control alongside this experimental animal, shows, contrary to the authors claim, distinct signs of metamorphosis, a reduction of the tail fin and instead of the larval gills mere stubs. It seems to me the number of Hirschler's positive experiments will have to be increased before they can be held against the negative experiments of Jensen, Huxley and Hogben, and myself.

As to Huxley's and Hogben's positive results [24] on the larvæ of *Salamander maculosa* and *triton*, quoted by Swingle against me, it should be stated that the method employed in these experiments is such as to permit of no conclusions whatsoever. In the first place, they did not use the first moulting, but the sizes of the gills as an indicator of metamorphosis. The gills may become reduced in size by the action of many factors different from metamorphosis, particularly by starvation. Since strong iodine solutions were used, it is almost certain that contrary to the authors' impression (quantitative measurements of the food intake were not made) the experimental larvæ fed less well than the controls. Secondly, nowhere in Huxley's and Hogben's paper can I find any statement indicating the size and stage of the larvæ at the beginning of the experiment. Yet if the larvæ were in an advanced larval stage any irritation as serious as that caused by iodine solutions would be sure to bring about precocious metamorphosis.

[24] Huxley, J. S., and Hogben, L. T., *Proc. Royal Soc.*, 1922, XCIII., 36.
[25] Hirschler, J., *Arch. Entwcklgsgsmech. d. Orgn.*, 1922, LI., 482.

It is evident that none of the observations according to which inorganic iodine does enforce the metamorphosis of salamanders can be accepted as correct at the present time.

That organic iodine compounds may enforce the metamorphosis of neotenous forms of salamanders has been claimed repeatedly and may be true, although the axolotl used generally in these experiments appears, for reasons stated above, to be an unreliable material. Jensen was the first one who studied, in an extensive manner, the influence of organic iodine-compounds upon the metamorphosis of axolotls. Where he left the problem it is still at the present time. In particular, Jensen deserves the credit for having recognized that the experiments with iodine could not advance the problem unless thyroidectomized larvæ are used. He was the first one who administered organic iodine compounds to thyroidectomized axolotls and stated [26] that thyroxine can be used directly by the organism without the intermediation of the thyroid. Swingle repeated these experiments [13, 21] using 3–5 di-iodo-tyrosine, a substance which Jensen [27] had found ineffective in the normal axolotl. Swingle reports that 3-5 di-iodo-tyrosine does enforce the metamorphosis of thyroidectomized axolotls. Both Jensen's and Swingle's experiments, however, should be taken with caution as far as the successful thyroidectomy is concerned. I am not certain at all that Swingle realizes that an axolotl possesses 4 thyroid glands, two main portions and two accessory ones. He mentions it nowhere and it is likely that only the main portions were extirpated. The accessory thyroid glands of *A. tigrinum* have a tendency to become very large and, after removal of the main portions, may enlarge considerably, so as to cause finally metamorphosis, as I observed in many larvæ of *A. tigrinum*. It is likely that Swingle's "thyroidless" axolotls were in the possession of two developing accessory glands; that an axolotl does not possess accessory glands I would be willing to believe only if sections through the entire region of the lower jaws, throat, and heart could be presented, since dissection, because of the hidden position of these accessory glands, may fail to demonstrate them. If the main thyroids are removed, it takes a long time before the accessories, in the event that they

[26] Jensen, C. O., *Compt. rend. Soc. Biol.*, 1921, LXXXV., 391.
[27] Jensen, C. O., *Compt. rend. Soc. Biol.*, 1920, LXXXIII., 315.

have been small, attain a size and structure capable of producing metamorphosis. But as shown in my iodine experiments, the feeding of iodine would greatly accelerate the elaboration of the hormone and, if the releasing mechanism is set active (which it was in Swingle's specimens, according to his own statements), metamorphosis may occur months before it takes place in the untreated controls. Swingle has observed his animals apparently only for 6 months; it would be important to know whether the untreated "thyroidectomized" animals did not finally metamorphose.

Swingle [13, 21] mentions also that 3–5 di-brom-tyrosine, when fed to thyroidectomized axolotl larvæ, is incapable of enforcing metamorphosis and thinks that this result is contrary to my own views on the rôle of iodine in the amphibian metamorphosis and in the thyroid hormone. Apparently he did not see the following statement, in which my views were summarized [10] (p. 114): "The views elaborated above are in no way contradictory to the fact that nevertheless, in a biological sense, iodine is an important and essential part of the thyroid hormone; if it were possible to substitute the iodine by any other substance without changing the reactivity of the hormone, biologically this would not make iodine less important, for it is the only substance which, by the mechanism actually available to the organisms, can be used in the manufacture of the thyroid hormone. Although chemically bromine or any other halogen may be able to substitute iodine without changing the chemical or even the physiological reactivity of the thyroid hormone, the organism is unable to use bromine, as shown by Swingle, and presumably the other halogens to make thyroid hormone." I have never claimed that the thyroid or any other organ can manufacture the thyroid hormone from bromine. Swingle has not touched, by his experiments, the real problem. This centers around the question whether the finished thyroid hormone could enforce metamorphosis if it contained bromine instead of iodine; Swingle did not employ such a product in his experiments.

As Swingle correctly states, the crux of the problem of thyroid function is now to find the organ or tissue or substance which plays the rôle of a releasing mechanism to the thyroid gland. I have intentionally refrained, in my previous papers, from forming

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any theories, aside from those directly suggested by the results of my experiments, as to the nature of the releasing mechanism; devoting pages to discussing assumptions and hypotheses does not materially advance the problem. We know, of course, that the hypophysis has something to do with the development and, possibly, with the function of the thyroid. I have made some experiments, to be published shortly, which seem to indicate that some unknown factor is located in the gills, in the absence of which the thryoid, although it develops in a normal manner, remains incapable of releasing the hormone. Swingle mentions one experiment which was intended to test the activity of the hypophysis of a neotenous axolotl by the grafting method. Although ultimately it may turn out that the hypophysis controls, in some way, the releasing mechanism, Swingle has so far contributed nothing to the solution of this problem.

Hence it is very evident that Swingle has not advanced, by a single step, the problem of neoteny and thyroid function beyond the stage at which my own researches left it.

SUMMARY.

1. Only in one, a sex-mature specimen, among 7 specimens of *Typhlomolge rathbuni*, is the thyroid completely absent; in the other 6 specimens rudiments of the thyroids are present.

2. The thyroid rudiments are undifferentiated epithelial cell masses located along the path of migration of the thyroid, typical for salamanders. They may contain a lumen, but never contain colloid and blood vessels.

3. *Typhlomolge* possesses 3 pairs of thymus glands.

4. The hypophysis is similar to that of other salamanders. But the partes tuberales are perhaps smaller than in the adult *A. opacum* and the pars nervosa is larger.

5. The postbranchial body resembles much that of other salamanders, but sometimes is shorter and lacking a lumen.

BREEDING EXPERIMENTS WITH CONFINED BREMUS (BOMBUS) QUEENS.[1]

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The biologist who attempts to give a complete account of the life-history and habits of the bumblebees of any part of the world, is generally confronted with the following difficulties: (1) He rarely, if ever, has the opportunity to study the beginning and early stages of a bumblebee colony, and (2) it is usually impossible for him to ascertain the biology of the less common species, because he is unable to secure their nests. The first attempts to overcome these difficulties were made by the Austrian zoölogist Hoffer ('82). This eminent bumblebee student confined a large number of *Bremus* queens in a museum at Graz, and thus was able to observe how the queen of *Bremus lapidarius* constructs her nest and the first egg-cell. However, none of these breeding experiments of Hoffer ('82, p. 413) produced a colony.

Better results along this line were obtained by the late F. W. L. Sladen ('12), who succeeded in rearing several colonies of *Bremus terestris*, a species which is very common in most parts of Europe. About the same time, similar experiments were carried out by the Danish biologist Lindhard ('12) with queens of *Bremus agrorum, distinguendus, hortorum, lapidarius, subterraneus, sylvarum,* and *terrestris*. With the exception of *Bremus hortorum* and *subterraneus*, at least one queen of each of these species started a nest, some of the resulting colonies later becoming self-supporting. In this country, Mr. Theodore H. Frison ('18) was equally successful in artificially rearing a colony of *Bremus auricomus*, a species concerning whose biology little was known up to that time.

I became interested in this subject during the summer of 1921 and decided to try similar artificial breeding experiments with our

New England species of *Bremus*. During the following spring and summer, about fifty queens belonging to eleven [1] of the thirteen New England species listed by Franklin ('12/13, pp. 190, 191) were captured in the Arnold Arboretum, within the city limits of Boston. After being confined for various periods of time, at least one queen of each of these species became broody and oviposited, but self-supporting colonies were only produced by six.[2] The names of these six species, and the number of colonies obtained from each, are listed in the accompanying table:

TABLE I.

	Number of
Species.	Colonies.
1. *Bremus bimaculatus* Cresson	2
2. *Bremus impatiens* Cresson	1
3. *Bremus perplexus* Cresson	1
4. *Bremus separatus* Cresson	2
5. *Bremus ternarius* Say	1
6. *Bremus vagans* Smith	2

Before discussing the methods which were used in these breeding experiments, it seems desirable to describe briefly those employed by the earlier workers. Hoffer ('82) supplied his queens with nesting material and plenty of fresh flowers. Each queen was probably confined in a separate box. At first, Sladen ('12) also confined each queen separately, giving her an artificial nest and a liberal supply of honey and pollen, but he was unable to get a colony started in this way. He then placed two queens (of the same species) in each box, and this method yielded better results. However, Sladen (p. 131) found that one of the queens always killed her companion about the time the eggs were laid, and that the victorious queen invariably deserted the nest, unless she was supplied with one or more workers. In order to avoid this killing of queens, Sladen (p. 132) modified the experiment by confining one queen with one or more workers in each box. This also proved successful, even when the workers were of a different species.

[1] *Bremus affinis, bimaculatus, borealis, fervidus, impatiens, pennsylvanicus, perplexus, separatus, ternarius, terricola,* and *vagans.*

[2] On June 13, several of these incipient bumblebee colonies were exhibited at a meeting of the Cambridge Entomological Club.

The methods employed by Lindhard ('12), differ in several respects from those of Hoffer ('82) and Sladen ('12). Lindhard (pp. 337, 338) used nest-boxes which were constructed as follows: Each nest-box consisted of two compartments of about 20x20x20 cm. each, one of which may be called the front compartment, or No. 1, and the other the rear compartment, or No. 2. On one side, compartment No. 1 had a glass pane to admit light. At the base, this compartment was provided with two flight-holes, *a* and *b*; *a*, communicating with the outside world, and *b*, with compartment No. 2. Both flight-holes could be opened and closed.

After lining compartment No. 2 with a layer of sod, about 5 cm. thick, Lindhard (p. 337) filled the interior with dry grass and the like. In some cases (cf. p. 341), a small paste-board box, filled with moss, and provided with a glass cover, was placed in the compartment instead of sod, and was surrounded with loose earth. With this arrangement, it was possible to place food in compartment No. 1 without disturbing the queen while she was engaged with her nest in the other compartment, and to keep the nest at a more uniform temperature.

As food, Lindhard (p. 337) provided a 50 per cent sugar solution and flowers, preferably those from which bumblebees were then obtaining pollen out in the open. After the queen had begun nest-building, the flight-hole was opened so that she could gather pollen from large bouquets which were put in the room in which the box was kept. As soon as the first worker emerged, the colony was placed out of doors where further development proceeded under normal conditions. In subsequent experiments, the queens were permitted to forage in the open shortly after they had begun nest-building. This method promised equally satisfactory results at the time Lindhard ('12) reported his work.

In his breeding experiments with *Bremus auricomus*, Frison ('18) confined two queens in one box, but, unlike Sladen (p. 131), found that they did not kill each other. As nesting material, Frison ('18, p. 44) supplied an old field-mouse nest in which he placed a honey-moistened lump of pollen. In addition to this, the queens were given a mixture of honey, rye-flour, and water.

The methods which were used in my own experiments, may be

described briefly as follows: Each nest-box was provided with a double cover, the lower being made of glass and the upper of wood or tar-paper. At one end of the box, a round hole, $\frac{1}{2}$ inch in diameter, which could be closed by means of a cork, served as a flight-hole. A small piece of honeybee foundation (wax), about an inch square, was then firmly pressed to the bottom of the nest-box near the opposite end. Around this piece of wax, a circular layer of cotton was placed as nesting-material. A tin can, about 3 inches in diameter and 2 inches high, from which the cover was first removed, was then put upside down over the honeybee foundation and cotton, after a hole, through which the queen could readily pass, had been made in the rim. Every two or three days, a fresh supply of pollen, obtained from two colonies of honeybees which had been especially secured for this purpose, was provided on the layer of wax within the cotton ring. Liquid food, consisting of about half water and half honey, was supplied daily in a porcelain dish, about $\frac{1}{2}$ inch high, outside of the tin can. In order to keep the nest-box as sanitary as possible, a small pile of dry sand was put in one of the corners of the box.

Not being acquainted with the methods employed by Lindhard ('12), at the time my experiments were carried out, I at first placed two queens (of the same species) in each nest-box, as was done by Sladen ('12) and Frison ('18). However, like Sladen (p. 131), I found that one of the queens invariably killed the other,[1] sometimes within a few minutes after they had been placed together, and that the victorious queen was frequently made useless for further breeding experiments by the loss of one or more antennæ, or legs.[2] I therefore only placed one queen in each nest-box in subsequent experiments, and furnished each one with from one to three workers, preferably of her own species. Whenever a worker died—a rather frequent occurrence, especially as long as there was no brood—another was substituted as soon as possible. Those bumblebee "nuclei" which belonged to species easily obtainable in or near Boston, were permitted to forage out

[1] As already stated, Mr. T. H. Frison ('18) found that two queens of *Bremus auricomus* behaved differently in this respect, but, judging from Mr. Frison's (p. 45) account, it seems probable that one of the queens was in poor health.

[2] These observations were made on about twenty queens of *Bremus bimaculatus, fervidus, impatiens,* and *vagans.*

of doors, whenever the weather was pleasant, provided eggs or larvæ were present in the nest. Shortly after the first worker emerged, the tin can was removed, and the young colony, after being provided with additional nesting material, was given complete liberty.

Several nuclei, instead of building the first cells on the honeybee foundation, started their nests on the floor of the box, outside of the tin can. The behavior of such nuclei was as follows: The queen and workers, with outstretched abdomens, nestled closely to the floor of the nest-box about a certain spot which they had cleared of all foreign matter. As a result of this behavior, the chosen spot was gradually (sometimes within a day or two) coated with a layer of wax, and at this place the first cell was built. On several occasions, I tried to discourage the bees from starting their nest outside the tin can, by placing sand on the spot which they had selected. However, this did not disconcert them, for they immediately began to push the sand aside, picking up the larger grains with their mandibles and carrying them to the periphery of the wax-covered area. This experiment was repeated several times with a nucleus of *Bremus bimaculatus*, but the bees could not be persuaded to start their nest under the tin can until a layer of soap was substituted for the wax which they had deposited.

Having given a general account of the methods used in rearing bumblebee colonies from confined queens, I shall now proceed to discuss my own experiments somewhat more in detail. In order to make these data as complete as possible, a brief account of what is known concerning the nesting habits of our New England species has been added, with the exception of those cases where this has already been done in a previous paper ('22*b*). Since all of these breeding experiments were carried out during the spring and summer of 1922, the year has been omitted from most dates, 1922 being understood, unless otherwise indicated.

Terrestris Group.

1. *Bremus affinis* Cresson.

From the latter part of May until the end of June, several queens of this species were confined in separate nest-boxes with

two or three workers. Although two of the queens constructed egg-cells and oviposited, the young larvæ died, apparently because they were not fed.

That it is possible to rear some, if not all, of our American *Bremus* species by the methods which were finally adopted by Lindhard ('12), is shown by the following incident, and another which will be discussed in connection with *Bremus vagans*. On May 26, a queen and two workers of *Bremus affinis* were confined together. Two days later, the queen constructed an egg-cell and oviposited, but on May 30, it became apparent that she had forsaken her brood. She was therefore set free from a third story window of one of the Bussey buildings, about noon on the following day, and her eggs and the two workers were turned over to another *affinis* queen. About five hours later, an *affinis* queen was noticed examining carefully several second story windows of the building referred to above, whereupon she mounted to the third story window from which the *affinis* queen had been liberated, and attempted to get in. I hastened upstairs, opened the window, and tried to catch her with my insect net, but missed her, and she flew away. About 11 A.M. on the following day (June 2), she again appeared at the third story window, but left as soon as I opened it, and, to my knowledge, did not return.

The nesting habits and disposition of *Bremus affinis* have been discussed in a recent paper ('22b), but the following incident seems worth recording. On June 2, a nest-box containing a queen, three workers, and an egg-cell of this species was accidentally jarred. Both, the workers and the queen immediately began to buzz angrily and rush out from beneath the tin can. In doing so, the queen accidentally encountered one of the workers, seized the latter and stung it to death.

II. *Bremus terricola* Kirby.

A queen of this species was confined on May 26, and a few days later three *terricola* workers were associated with her. Shortly after the introduction of the workers, the queen oviposited, and on June 16, the nest contained several small larvæ. About two weeks later (July 4), two exceedingly small *terricola* workers were noticed on the sand pile. They were unable to crawl, and had probably been released from their cocoons by the

queen or workers and then dragged to the sand pile. No other brood being present in the nest, this nucleus was combined with another *terricola* colony which had been taken on the preceding day.

The nesting habits of this species have been dealt with in another paper ('22*b*).

Borealis GROUP.

I. *Bremus borealis* Kirby.

Four queens of this species, captured in or near the Arnold Arboretum, were experimented with. As no *borealis* workers could be obtained in the vicinity of Boston, two workers of *Bremus fervidus* were given to queen No. 1 (confined May 29), and three workers of *Bremus impatiens* to queen No. 2 (confined June 6), but neither one of the queens would coöperate with the foreign workers. On June 25, queen No. 1 was found dead in the nest, whereupon the workers of both queens were liberated, neither queen having started a nest. A somewhat different method was then resorted to. On June 26, about a dozen cocoons of *Bremus impatiens* from which workers were just beginning to emerge, were given to *borealis* queen No. 2. She immediately adopted both, the cocoons and the workers, and on June 28 constructed an egg-cell and oviposited. Two days later, this mixed colony was permitted to forage out in the open, after a small notch had been made in one of the wings of the queen. When the nest was examined on the following day, the queen was missing and did not return.

Borealis queen No. 3, captured July 2, was confined with sixteen worker cocoons of *Bremus impatiens*, which she adopted at once. On the following day, she built an egg-cell and oviposited. By July 5, four workers of *Bremus impatiens* had emerged, and this *borealis-impatiens* colony was also given complete liberty; but eight days later, the queen was found dead in a corner of the nest-box, probably as a result of an encounter with the workers.

Queen No. 4 was confined on July 8 with fourteen worker cocoons of *Bremus fervidus*, which were adopted immediately. She laid a batch of eggs on the following day, and another on

July 11. Meanwhile several *fervidus* workers had hatched, and, beginning July 13, the colony was allowed to forage in the open. For a time, this *borealis-fervidus* colony seemed to get along very well, but on July 19, it was noticed that the *fervidus* workers kept the *borealis* queen from the comb most of the time by daubing her with honey, a habit which has been described in another paper ('22a). In spite of this treatment, the queen lingered about the nest until August 18, when she died. During the first part of August, several *fervidus* males hatched in this nest, but no adult *borealis* were obtained from any of these mixed colonies.

As has been pointed out in another paper ('22b), practically nothing is known concerning the nesting habits of this species.

Pratorum GROUP.

I. *Bremus bimaculatus* Cresson.

After losing several *bimaculatus* queens through dueling, a queen of this species was confined alone on May 20. Two days later, a *bimaculatus* worker was given to her, but she squirted the latter with faeces,[1] and showed her hostility in other ways. On the following day, another *bimaculatus* worker was substituted for the first. With this second worker she soon became friendly, and by the next morning a honey-pot and a cell containing eggs were present in the nest. On May 26, two more *bimaculatus* workers were added to this nucleus. The first batch of larvæ— twelve in number—grew rapidly and began spinning their cocoons about June 7, and the first adult—a male—emerged on June 18. The bees which hatched from the remaining eleven cocoons, as well as those which emerged later, were likewise males. It is evident, therefore, that this *bimaculatus* queen had not been fertilized the preceding fall, and that, in some instances, bumblebee males may be produced as early in spring as workers, a fact which has been overlooked by other bumblebee students (cf. Dahlbom ('32, pp. 9, 10), Schmiedeknecht ('78, pp. 317, 320, 323), Hoffer ('82/83, p. 15), Wagner '07, p. 126), Sladen ('12, p. 49), and Stellwaag ('15, pp. 466, 467)).

[1] This method of warfare is also employed by the queens and workers of other American species, *e.g.*, *Bremus impatiens*. In Europe, Wagner ('07, p. 82) observed a similar behavior in the case of *Bremus variabilis*, a queen of this species squirting the liquid for a distance of more than 35 cm.

Another *bimaculatus* queen and two workers were confined together on May 26. The first eggs were laid on June 5, and the first worker emerged on June 29, whereupon the colony was given complete liberty. On July 17, this colony consisted of the queen and 23 workers, a number of queens and males being produced later. The colony had completely died out by August 15.

The nesting habits and disposition of this species have been dealt with in two other papers ('22, '22b).

II. *Bremus impatiens* Cresson.

After several queens of *Bremus impatiens* had killed each other, a queen of this species was confined alone on May 20. By May 27, she had constructed an egg-cell and oviposited, and on the following day she proceeded to build a honey-pot, all of this work being done without the assistance of workers. On May 31, June 2, and June 3, respectively, three workers of *Bremus impatiens*—the first obtainable—were given to her, and by June 4, another honey-pot and two additional batches of eggs were present in the nest. On June 9, the first batch of larvæ—eight in number—were almost full-grown, and ten days later the first worker emerged, whereupon the flight-hole was left open permanently. When this colony was examined on August 15, it consisted of the queen, 122 workers, and a considerable quantity of brood. The colony broke up toward the end of September, after having produced a large number of males and young queens.

The nesting habits and temper of this species have been discussed in several other papers ('22, '22a, '22b).

III. *Bremus perplexus* Cresson.

A queen and two workers of this species were confined on June 6, and another queen and three workers on June 11. Both nuclei began nest-building on the day on which they were confined, but on June 17, queen No. 2 and one of her three workers were found dead in the nest, whereupon the remaining two workers and brood were given to queen No. 1. The larvæ grew rapidly, and on June 29,—twenty-three days after queen No. 1 was confined—the first worker emerged.[1] Several others hatched during

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[1] This confirms the observations of Sladen ('12, p. 31) and Frison ('18, p. 47), who found that it takes from 22 to 25 days for the workers to emerge, from the time the eggs are laid. Hoffer's (82–83, p. 28) claim that the development of the workers, from egg to adult, takes a month, is therefore incorrect.

the next few days, and on July 1, the colony was given its liberty. In order to give it a better start, about twenty cocoons of *Bremus impatiens* were placed in the nest. The workers of the two species showed no hostility toward each other, and everything went well until July 14, when the *perplexus* queen was found dead in the nest. Like *borealis* queen No. 3, she probably was killed by the *impatiens* workers. During the first half of August, this *perplexus-impatiens* colony produced several males of both species, but by August 20, the colony had completely broken up.

What is known about the nesting habits of *Bremus perplexus*, we owe to Franklin ('12/13, pp. 347–348). Some years ago, this author took two nests in early August, in Vermont. Both nests were situated in the walls of houses, and were made of wool. Qne of the nests contained 5 queens, 1 male, and 9 workers; and the other, 8 queens and 33 workers.

In the vicinity of Boston, *Bremus perplexus* is very rare. Judging from the early appearance of the workers (the first one was taken on May 28), some queens of this species must appear as early as May 1, and most nests are probably started during that month. The sexual forms seem to be produced chiefly during July and August. The nests probably break up in September.

Regarding the disposition of *Bremus perplexus*, Franklin (p. 348) has the following to say: "This is the gentlest and least ready to sting of all the bumblebee species which I have had to deal with in the living condition. This seems peculiar, as *B. vagans*, which seems to be its nearest ally, is exceedingly ferocious." I have already ('22b) taken exception to the last part of this statement. According to my observations, *Bremus perplexus* and *Bremus vagans* are similar in disposition, both species being comparatively gentle.

IV. *Bremus ternarius* Say.

Of this species, two queens were taken on *Rhododendron*, in the Arnold Arboretum, on June 6, and June 8, respectively. Besides having lost much of her pile on the dorsal side, *ternarius* queen No. 2 had a very distended abdomen, suggesting that she probably had already started a colony. She was therefore set free a few minutes after she was captured with the hope that she might furnish workers for *ternarius* queen No. 1. As queen No. 1

showed no interest in the nesting material, three workers of *Bremus bimaculatus* were placed in her nest-box on June 14, but she would have nothing to do with them, and three days later, two small workers of *Bremus impatiens* were substituted for the three *bimaculatus* workers. With these two workers, the *ternarius* queen made friends and a few days later began nest-building. The first batch of eggs was laid about June 21, and the first *ternarius* worker hatched on July 14. By July 16, six more workers had emerged. The two workers of *Bremus impatiens* were then removed, and the young *ternarius* colony was left to shift for itself. By August 13, the number of workers had increased to seventeen, and a few weeks later, several newly-hatched *ternarius* males were present in the nest. At the beginning of September, the queen showed signs of becoming feeble, and on September 10, disappeared from the nest. The last workers died during the first week of October.

Very little is known concerning the nesting habits of *Bremus ternarius*. During the summer of 1863, Putnam ('64) took a nest[1] of this species at Bridport, Vt., on the borders of Lake Champlain. It was situated either under an old stump or under the clapboards of a house.

In the vicinity of Boston, *Bremus ternarius* is exceedingly rare. The queens, like those of *Bremus vagans*, seem to leave their winter quarters comparatively late in the spring. Most nests are probably started between the 15th of May and the 15th of June. If this assumption is correct, the first workers ought to appear shortly after June 1. As in most other New England species, the males and queens are probably produced chiefly during August and September.

Putnam (p. 99) states that *Bremus ternarius* is far more savage than *Bremus fervidus*, the latter species, according to this author, being "of quite a gentle disposition." However, I found both of these species to be extremely vicious. In other respects, the behavior of *Bremus ternarius* reminds one very much of that of *Bremus perplexus* and *Bremus vagans*.

[1] The other nest which Packard ('64) and Putnam ('64) considered as belonging to *Bremus ternarius*, according to Franklin ('12/13, pp. 444-445), was probably a nest of *Bremus rufocinctus*.

V. *Bremus vagans* Smith.

As in the case of *Bremus bimaculatus* and *Bremus impatiens*, several queens of this species were at first lost through dueling. The two queens from which self-supporting colonies were obtained, were confined—each with three workers—on June 8, and 11, respectively. Within a week, both nuclei had begun nest-building, and by the end of June the first batches of larvæ were spinning their cocoons. The first worker of nucleus No. 1 hatched on July 13, and the first one of nucleus No. 2, on July 14, whereupon both nuclei were given continuous liberty. The two colonies prospered and did not break up until the latter part of September, each having produced a number of queens and males.

As already mentioned in connection with *Bremus affinis*, a confined bumblebee queen, if liberated, may return to an artificial nest after she has oviposited in it. From the following incident, it will be seen that *Bremus vagans* is no exception to this rule. On June 22, the weather was exceptionally pleasant, and *vagans* nucleus No. 2 having small larvæ, the flight-hole of the nest-box was opened at about 9 A.M., in order to give the bees a chance to forage. When the nest was examined at noon, the queen, as well as the workers, had disappeared. At 2 P.M., none of the bees had returned, and, believing they had forsaken the brood, the nest-box was removed with the intention of turning the young larvæ over to *vagans* nucleus No. 2. However, about 5 P.M., *vagans* queen No. 1 was found eagerly searching about the place where the nest-box had been. She was captured, and upon being placed in the nest-box, quickly went to her brood. The workers did not return, and three others were substituted on the following day.

The nesting habits and disposition of *Bremus vagans* have been discussed in two recent papers ('22, '22*b*).

Auricomus GROUP.

I. *Bremus auricomus* Robertson.

This is one of the two species which I was unable to obtain in the vicinity of Boston. All that is known concerning the nesting habits of *Bremus auricomus*, we owe to the efforts of Mr. Theodore

H. Frison ('17, '18, '21). In addition to the colony which he reared artificially, Mr. Frison ('17, '21) had under observation several nests of natural origin, one of which was taken on September 6, 1917. It was situated in a hollow cement block in the foundation of a small cabin, and contained 3 young queens, 3 males, and 15 workers—10 living and 5 dead—besides several others which were out foraging when the nest was taken. Another nest, examined July 26, 1919, at Clyman Junction, Wis., was situated about 1½ ft. below the surface of the ground, and contained the old queen, 12 workers, 15 eggs, and some larvæ and pupæ of *Bremus auricomus* as well as a disabled *Psithyrus laboriosus* queen. In addition to these three colonies, Mr. Frison ('17) had under observation another which was started in an artificial nest which had been placed in a clay embankment.

According to Mr. Frison ('18), *Bremus auricomus* is rather gentle in disposition. Concerning the colony which he took on September 6, 1917, he has the following to say: "The bumble-bees were very docile when the nest was removed, for instead of flying angrily from the nest, the most they did was to run excitedly about on the comb and buzz loudly."

Fraternus GROUP.

I. *Bremus rufocinctus* Cresson.

As in the preceding case, I was unable to obtain queens of this species in the vicinity of Boston. Comparatively little is known about the nesting habits of *Bremus rufocinctus*. According to Franklin ('12/13, pp. 444–445), Putnam ('64) took a nest of this species at Bridport, Vt., in September, 1863. It was probably situated under the clapboards of a house, about eight feet from the gound, and contained 28 adult bees and 35 cells with young.

Judging from Putnam's (p. 99) account, *Bremus rufocinctus* is one of the more savage species.

II. *Bremus separatus* Cresson.

On May 15, a queen of this species which had been captured at Peabody, Mass., was turned over to me by Dr. L. H. Taylor. She had lost a part of one of her antennæ and, although given a *separatus* worker, refused to take any interest in the nesting material. She was found dead in the nest-box on June 9.

Queen No. 2 was taken on June 3. Having lost both of her antennæ, she took little interest in life and died five days later.

Queens No. 3 and No. 4 were taken in the Arnold Arboretum on June 8, and June 16, respectively. They were confined separately, and each one was given three workers. Both of these nuclei at once started nest-building, and toward the end of June each had large larvæ. The first workers emerged on July 9, and 11, respectively, whereupon both colonies were given their liberty. A few days later, queen No. 4, returning from a foraging trip, by mistake entered a nest of *Bremus affinis* and was stung to death. Her brood and workers were given to *separatus* colony No. 3. This colony prospered and produced a number of males and queens in August, but had completely died out by September 10.

According to Putnam ('64), *Bremus separatus* builds its nests "under old stumps and in other situations similar to those in which the nests of *B. fervidus* are found."

In regard to the disposition of *Bremus separatus*, Putnam (p. 101) has the following to say: "This species is nearly as ferocious, on being disturbed, as *B. ternarius*," a statement which is corroborated by my own experience.

Dumoucheli GROUP.

I. *Bremus fervidus* Fabricius.

After losing several queens of *Bremus fervidus* by dueling, a queen of this species was confined alone on May 24, but she refused to start a nest. On June 2, three *fervidus* workers were associated with her, and three days later the nest contained two honey-pots and a closed egg-cell, but the larvæ which hatched from the eggs died, apparently because they were not fed by the adults. Several other *fervidus* nuclei which were started later, likewise paid no attention to their larvæ.

The nesting habits and disposition of *Bremus fervidus* have been discussed in several recent papers ('22, '22a, '22b).

II. *Bremus pennsylvanicus* De Geer.

A queen of this species was confined on May 29, and six days later, three workers of *Bremus fervidus* were given to her, but she remained restless and would have nothing to do with them.

Another *pennsylvanicus* queen was therefore put in her place on June 5. Although hostile to the *fervidus* workers, queen No. 2 constructed an egg-cell and oviposited on June 11. But, as in the case of the *fervidus* nuclei, the larvæ were not fed and died shortly after hatching. Both *Bremus fervidus* and *Bremus pennsylvanicus* are Pocket-makers, *i.e.*, they feed their larvæ, at least those of the workers,[1] through one or more pockets which they make at the side of each group of larvæ. On returning from the field, the foraging bee deposits its load of pollen directly into these pockets, through which the latter reaches the larvæ. It seems probable, therefore, that the Pocket-makers let their worker larvæ die, whenever they cannot feed them in the usual way. If this supposition is correct, it will be impossible to rear colonies of the Pocket-makers from confined queens, unless the latter are permitted to collect pollen from flowers.

Since the methods employed in rearing colonies of other species yielded no results in the case of *Bremus fervidus* and *Bremus pennsylvanicus*, and as I was anxious to obtain a colony of the latter species, a different method was resorted to. On June 26, about a dozen cocoons of *Bremus impatiens* were given to *pennsylvanicus* queen No. 2, which she adopted immediately. She showed no hostility toward the young workers which emerged, and two days later constructed an egg-cell and laid a batch of eggs. On July 2, this mixed colony was placed out of doors so that the workers could forage. Everything went well until July 6, when the queen was found dead in the nest, having probably been killed by the *impatiens* workers.

On July 26, a third *pennsylvanicus* queen was confined with a *pennsylvanicus* worker and sixteen cocoons of *Bremus fervidus*, and on August 2, another *pennsylvanicus* worker was added to this nucleus. The cocoons were adopted immediately, as were the *fervidus* workers which hatched from them. On August 3, the queen built an egg-cell and oviposited, and two days later this *fervidus-pennsylvanicus* colony was given its liberty. *Pennsylvanicus* worker No. 1 did not return, but No. 2 and several of the *fervidus* workers brought in one load of food after another.

[1] The queen and male larvæ of *Bremus fervidus*, and probably also those of *Bremus pennsylvanicus*, are fed, at least toward the end of their development, like the larvæ of those bumblebees which do not feed their larvæ through pockets.

For several days, everything went well, but on August 10, it was noticed that the *fervidus* workers were daubing the *pennsylvanicus* queen and worker with honey, a habit which has been referred to before. On the next day, the *pennsylvanicus* worker failed to return, but the queen, although her pile was constantly soaked with honey, lingered about the nest until August 21, when she disappeared. A few days later, several *fervidus* males hatched in this colony, but no adults of *Bremus pennsylvanicus* were obtained from any of these mixed colonies.

The nesting habits of *Bremus pennsylvanicus* have been described by Franklin ('12/13) Howard ('18), and Frison ('16, '17, '18, '21). Judging from the data published by these authors, the nests are usually situated on the surface of the ground, but occasionally also in the ground, or in birds' nests. The largest nest taken by Franklin (p. 405) contained 1 queen, 23 males, 53 workers, and 78 cells with larvæ in them, of which 18 were queen cells.

In the vicinity of Boston, *Bremus pennsylvanicus* is comparatively rare. The queens are the last to appear in spring, the first one in 1922 being seen on May 29, and the first worker on July 22. Most nests are probably started in June. A number of males of this species were taken in September, and therefore the colonies, like those of *Bremus fervidus*, probably do not break up until the latter part of September or the beginning of October.

According to Mr. T. H. Frison ('17, '18) and Mr. Court W. Ranslow (cf. Howard, '18), the workers of *Bremus pennsylvanicus* are rather vicious. After they had oviposited, this was also true of the *pennsylvanicus* queens used in my breeding experiments. On several occasions, they seized my forceps, tried to sting them, and clung to them so tenaciously that they could be lifted out of the nest-box.

While these experiments were in progress, a number of other observations were made which will be presented in another paper.

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